Novel Clinical and Etiopathogenetic Findings in Pseudoxanthoma Elasticum

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Thesis submitted to fulfill the requirements
for the degree of Doctor in Medical Sciences

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From 1-10-2003 until 30-09-2007, Olivier Vanakker was an Aspirant of the Fund for Scientific Research – Flanders.

This thesis is dedicated to my grandparents.
Amicitia perpetua

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List of abbreviations

ABC	ATP-binding cassette
AFI	autofluorescence imaging
A(R)MD	age-related macular degeneration
AS	angioid streaks
ATP	adenosine 5'-triphosphate
BCVA	best corrected visual acuity
BrM	Bruch membrane
BMP	bone morphogenetic protein
CAD	coronary artery disease
CC	choriocapillaries
cSLO	confocal scanning laser ophthalmoscope
C(T)	comet (tail)
cMGP	carboxylated matrix gla protein
cOC	carboxylated osteocalcin
CTD	connective tissue disease
CVA	cerebrovascular accident
dHPLC	denaturing high-performance liquid chromatography
DNA	desoxyribonucleic acid
dpMGP	desphosphorylated matrix gla protein
ECM	extracellular matrix
ELISA	enzyme-linked immunosorbent assay
ELN	elastin
ERG	electroretinography
GAS-6	growth-arrest specific protein 6
GGCX	gamma-glutamylcarboxylase
IC	intermittent claudication
IFN-γ	interferon γ
IHC	immunohistochemistry
IRI	infrared imaging
LAC-PXE	localized acquired cutaneous PXE
LTBP	latent TGF-β binding proteins
MAGP	microfibril-associated glycoprotein
MGP	matrix gla protein
MLPA	multiplex ligand probe amplification
MRP	multidrug resistance protein
MVP	mitral valve prolapse
NBF	nucleotide binding fold
NEM-SG	N-ethyl maleïnide S-glutathione
NMD	nonsense-mediated decay
OMIM	online Mendelian in man
OC	osteocalcin
OPN	osteopontin
PAD	peripheral artery disease
Pd'O	peau d'orange
pMGP	phosphorylated matrix gla protein
POHS	presumed ocular histoplasmosis syndrome
PXE	pseudoxanthoma elasticum
RFI	red-free imaging
RPE	retinal pigment epithelium
RNA	ribonucleic acid
RT	room temperature

TE	tropoelastin
TGF-β	transforming growth factor β
TM	testicular microlithiasis
TMD	transmembrane domain
TNF-α	tumor necrosis factor α
TOAST	trial of ORG 10172 in acute stroke treatment
ucMGP	uncarboxylated matrix gla protein
ucOC	uncarboxylated osteocalcin
VEGF	vascular endothelial growth factor
VK	vitamin K
VKOR	vitamin K epoxide reductase

Preface
PSEUDOXANTHOMA ELASTICUM: History of a triad disorder

"Nous dirons avec lui [M. Chambard] *que ce qu'il faut trouver maintenant, c'est la cause première, le moteur du processus xanthélasmique. C'est la ce qu'il faut connaître avant de pouvoir classer le xanthélasma."*

Felix Balzer, 1884

From ancient mythology to present catholic believes, triads have always been a symbol for the mysterious, stirring ones imagination. The Ptah-Sekhmet-Nefertem Memphis triad, the resistance movement against Chinese Manchu emperors, the Divine Trinity and so many other examples all reflect how they intrigued mankind throughout the centuries. Often, it was or is not clear why three apparently different entities were joined, if not that they could perform different, yet related functions contributing to a purpose akin.

In many ways, pseudoxanthoma elasticum (PXE) – a classic example of a triad disease – reflects the mystery and ambiguous character of the ancient and modern triads. Perhaps this is the reason why it has fascinated researchers throughout the world and stimulated them to try and unravel the enigmas that PXE lays in front of them. And although some puzzles of PXE have been solved, many more remain subject of intensive exploration.

As H.B. Stowe already said, the past, the present and the future are really one: they are today. So, before looking at the present and taking a glance at the future, allow me to elaborate on the history of PXE. Although probably as old as mankind, the first descriptions of PXE by Rigal and Balzer date from 1881 and 1884 respectively [1, 2]. D. Rigal is credited with the first description of the skin lesions, while Felix Balzer provided the first autopsy report and grouped

the disorder with the xanthomatoses. The disorder was however given its current name by Jean-Ferdinand Darier, who in 1896 described the histological changes of the elastic fibres, which remain to date the hallmark of PXE [3]. As such, Darier was the first to perceive PXE as a separate, non-xanthomatous entity.

In 1929, 48 years after the initial description by Rigal, a Swedish ophthalmologist, Ester Grönblad and her colleague James Strandberg, a dermatologist made the first unequivocal connection between the skin and eye manifestations of PXE [4, 5]. As descriptive medical science in the late nineteenth and early twentieth century tended to name new discoveries after their discoverers, the Grönblad-Strandberg syndrome was born. Angioid streaks, the most prominent feature of the PXE retinopathy, were however already described in 1889 by Robert W. Doyne, an English ophthalmologist, while the term “streaks” was coined by a German born American ophthalmologist, Herman J. Knapp, in 1892.

The full triad of PXE was completed in the 1940s, when René Touraine and collegues studied the cardiovascular aspects of PXE. It has remained essentially unchanged ever since [6, 7].

PXE has for a long time been thought of as a primary disorder of elastic fibres, which was reflected by the screening of numerous candidate genes (elastin, fibrillin-1, fibrillin-2) encoding structural components of these fibres or related enzymes [8]. The mapping of the PXE gene to chromosome 16p13.1 was followed by the surprising finding that the causal gene for PXE codes for a transporter protein which had no apparent connection to the extracellular matrix [9-15]. This finding was intriguing, as it raised novel, exciting pathophysiological hypotheses and changed our view on PXE as a connective tissue disorder towards a metabolic disease with important implications for connective tissue homeostasis.

In 1884, Balzer already emphasized the importance of unravelling the pathophysiological mechanisms, “the motor”, which drives this complex disorder. Today, over one hundred years later, this quest remains more up to date than ever and represents the motor for this PhD thesis. While attempting to contribute to the better understanding of the mechanisms leading to PXE, it is however in first instance a humble tribute to the genius and magnificence of human (molecular) biology.

Chapter 1
Introduction

"My scientific studies have afforded me great gratification; and I am convinced that it will not be long before the whole world acknowledges the results of my work."

Gregor Mendel, 1865

1.1 Fundamentals of human genetics

Genetics is the dynamic science dealing with the mechanisms of hereditary transmission and the variation of hereditary characteristics – either physiological or pathological – among (similar or related) organisms. Although considered to be a relatively young branch of medicine and biology, it is primarily based on principles which were already published in 1866 by Gregor Mendel. This Austrian monk was the first to describe the basic principles of heredity by his experiments with pea plant characteristics. Only a few basic principles on patterns of transmission, terminology and molecular defects – necessary to fully understand this thesis – will be briefly touched.

1.1.1 Clinical genetics

1.1.1.1 Modes of inheritance

The three basic patterns of inheritance of Mendelian disorders are autosomal dominant, autosomal recessive and X-linked. Y-linked inheritance is rare and only mentioned for the sake of

completeness. Non-Mendelian inheritance patterns are beyond the subject of this thesis and will not be discussed.

1/ **autosomal dominant** inheritance occurs when one mutated gene copy (or allele) is sufficient to develop clinical disease. As the patient passes on one of both alleles to his offspring, children have a 50% risk of being affected. As a result, each generation of the family usually has affected individuals, unless it concerns a novel mutation occurring for the first time in the patient.

2/ **autosomal recessive** inheritance requires both alleles to be mutated before disease can occur. As the patient has received one mutated allele from each of his parents, both parents are obligate carriers (or heterozygotes) of one mutation. Conversely, as one allele is passed on to the patients' offspring, they also are obligate carriers. The risk for offspring to develop clinical disease is limited, unless there is consanguinity. Consanguinity increases the likelihood of both partners being heterozygous for the same mutation, hence increasing the risk that their children will inherit two mutated alleles. Typically, the two alleles will then carry the same mutation (homozygosity).
The term **pseudodominant** refers to the situation where an affected individual of an autosomal recessive disease has children with a heterozygous carrier. The pedigree of these families mimics autosomal dominant inheritance.

3/ **X-linked** inheritance involves disorders caused by mutations in a gene located on the X chromosome. These disorders can be either dominant or recessive. As males have only one X chromosome, next to a Y chromosome and females have two X-chromosomes, the X-linked recessive disorders are mostly seen in males with little or no symptoms in females. Exceptions to this rule can occur due to skewed X inactivation or lyonisation. X-linked dominant disorders can occur both in females and males, with a ratio of 2 to 1, reflecting the ratio of X chromosomes. The latter are also often lethal in males.

4/ **Y-linked** inheritance is extremely rare because of the few genes on the Y-chromosome; only male to male transmission occurs.

1.1.1.2 Terminology

While the human genome, our complete DNA sequence containing the entire genetic information of an individual, has not yet revealed its last secret, we have been confronted with its huge complexity. As often in science, genetics added to this complexity by creating its own terminology – by outsiders often considered as "magical" or "witch craft" – of which the most common terms, which are used throughout this thesis, are explained below.

A **gene** is a sequence of DNA, necessary for the production of a functional product. It is in fact the "unit of heredity". The human genome contains approximately 30.000 genes, located on 23 pairs of chromosomes. Each of these genes occurs twice in the genome; these copies, which are identical and positioned in the same location (or **locus**) are called **alleles**. **Transcription** involves

the synthesis of a single-stranded RNA molecule from a DNA template in the cell nucleus, while **translation** is the synthesis of a polypeptide from this mRNA template.

A **phenotype** comprises the observed biochemical, physiological and morphological characteristics of an individual or, in a more limited sense, the abnormalities resulting from a particular mutant gene.

A **phenocopy** is a mimic of a phenotype that is usually determined by a specific genotype, produced instead by the interaction of some environmental factor with a normal genotype.

A **genotype** is the genetic constitution of an individual or, more specifically, the two alleles present at one locus.

Pleiotropism of a gene reflects to the contributions of apparently unrelated phenotypical features resulting from mutations in a single gene.

1.1.2 Molecular genetics

The elementary techniques in molecular genetics – gene detection techniques, DNA amplification and sequencing – have as common goal the detection of one or two causal mutations (or genotype), responsible for the combined clinical signs and symptoms of a patient (or phenotype). While the technical details of these procedures will be explained in chapter two, we will here briefly review the different types of genetic defects which can lead to disease.

1/ A **missense** mutation is a single DNA base substitution resulting in a codon (or group of three base pairs) specifying an amino acid different from the wild type sequence.

2/ A **nonsense** mutation is a single base pair change resulting in a stop codon, ending transcription prematurely. Most often, the shortened transcript will be degraded in a process called nonsense-mediated decay (NMD).

3/ A **splice site** mutation interferes with the physiological removal of introns in the generation of mature mRNA (or splicing) by changing the binding site for enzymes necessary for splicing.

4/ A **deletion** or loss of one or more basepairs.

5/ An **insertion** or addition of one or more basepairs to the DNA sequence.

6/ A **frameshift** mutation involves a deletion or insertion that is not an exact multiple of 3 basepairs, changing the reading frame of the gene. All coding regions 3' of the mutation will be read completely different, usually soon encountering a stop codon.

Besides the above mentioned types of mutations, certain base pair variations in the genetic code are considered as physiologic alternatives or polymorphisms. Although such a polymorphism cannot be attributed to cause a disease by itself, many of these have been shown

to have an effect on the expression and regulation of the gene they occur in and as such have relevance in human (patho)physiology.

1.2 Connective tissue disorders

1.2.1 The connective tissue

The connective tissue, as the name implies, is a basic type of tissue providing structural and metabolic support for other tissues and organs throughout the body. Beside the loose connective tissue, also bone, cartilage and blood are considered part of this supportive tissue.

The uniqueness of connective tissue, compared to the other tissues of the body, lies in its composition of a diverse set of constituents – cells, fibres, blood vessels – scattered around in an extracellular matrix (ECM – Figure 1). Four types of macromolecules can be distinguished in the ECM: collagen, elastin, glycoproteins and proteoglycans. Each of these are produced by cell types specific for the various tissues. Among those, the fibroblast is the most important to maintain structural integrity of the ECM. It is however thought that over 2500 proteins are involved in ECM composition.

Although its presumed composition and historical function as biological packing material between cells and other tissues makes believe that the connective tissue is a static structure, it has become clear in recent years that this perception could not be further away from the truth. Indeed, as its complexity becomes more apparent, evidence is emerging that the ECM is a dynamic structure, playing a crucial role in the physiology of cells and tissues and influencing – through various signal transduction pathways – several biological processes such as cell growth, differentiation, migration, development and survival.

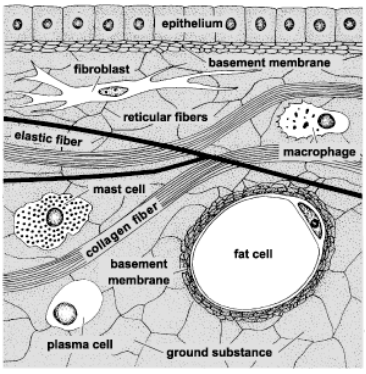

Figure 1
Simplified overview of the major components of the connective tissue
Adopted from McGraw-Hill Encyclopaedia of Science and Technology

1.2.1.1 Elastin and elastic fibres

The elastic fibre system forms a network responsible for the resilience and elasticity of various tissues. It consists of interconnecting fibres of varying diameter, containing two distinct components: elastin, a well-characterized connective tissue protein and elastin-associated microfibrils, the components of which include fibrillin, a microfibril-associated glycoprotein. The

biology of elastic fibres is complex because of its multiple associated molecules (Table 1), tightly regulated developmental pattern of deposition, multi-step assembly, unique elastomeric properties and influence on cell phenotype. Hence, what follows is only a brief summary of knowledge on elastic fibre biology, with abstraction of different facets of its complexity.

Molecule	Function
Fibrillin-1	Architectural function in EC microfibrils
Fibrillin-2	Architectural function in EC microfibrils
Fibrillin-3	Architectural function in EC microfibrils
MAGP-1	Unknown
MAGP-2	Unknown
LTBP-1	Structural component of TGF- β1
LTBP-2	Production and degradation of ECM
LTBP-3	Production and degradation of ECM
LTBP-4	Production and degradation of ECM
Decorin	Cell cycle regulation
Biglycan	Unknown
Versican	Unknown
MFAP-1	Cell division
MFAP-3	Unknown
MFAP-4 (MAGP-36)	Unknown
Tropoelastin	Initial translation product of *ELN*
Lysyl oxidase (LOX)	Elastic fibre cross-linking
LOXL	Cross-linking enzyme
LOXL2	Cross-linking enzyme
LOXL3	Cross-linking enzyme
BigH3 (keratoepithelin)	Corneal adhesion protein
Fibulin-1	Organogenesis
Fibulin-2	ECM constituent
Fibulin 4	Elastic fibre formation
Fibulin-5	Vascular development and remodelling
Emilin-1	Inhibitor of TGF-β signalling
Emilin-2	Unknown
Elastin-binding protein	Elastin chaperone protein
Vitronectin	Cytolysis, blood coagulation
Amyloid	
Collagen VIII	Component of Descemet membrane
Collagen XVI	ECM interactions
Endostatin	Angiogenesis inhibitor
Collagen VI	Anchoring function

Table 1
Reported microfibril and elastic fibre associated molecules
Adopted from Kielty et al. [16]

a/ elastic fibre organisation

Ultrastructural studies have provided insights into the organisation of the microfibrils and elastic fibre core, which will be briefly presented.

1/ The **microfibrillar component** of elastic fibres consists of fibrillin-rich microfibrils which show a 56 nm beaded periodicity (Figure 2). Calcium plays a key role in microfibril organisation through its influence on this beaded periodicity. Characteristic for this organisation is the octagonal structure of up to eight fibrillin molecules.

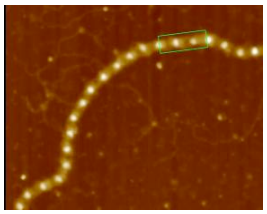

Figure 2
Atomic force microscopic image of microfibril, illustrating bead-to-bead periodicity
Adopted from Kielty et al. [16]

Fibrillins are highly homologous glycoproteins, of which 5 isoforms have been described with the same overall arrangement of repetitive domains. The expression pattern of fibrillins is distinct but overlapping [17]. Time of expression varies from early development to adult life, reflecting different functions in elastic fibre formation and/or maintenance [18].

Apart from fibrillins, **microfibril-associated glycoprotein 1** (MAGP-1) is associated with virtually all microfibrils and may be important for structural integrity. In contrast, MAGP-2 may have a function related to cell signalling during microfibril assembly and elastinogenesis. Besides the latent TGFB-binding proteins (LTBP), important for TGF-β targeting, several other microfibril-associated proteins and proteoglycans have been identified, many of which have an as yet unknown function (Table 1).

2/ The **elastic fibre core** has been shown not to be amorphous – in contrast to early descriptions – but instead to consist of laterally packed, thin ordered filaments [19].

b/ elastic fibre assembly

1/ **Microfibrils** assemble close to the cell surface in a process which is not fully understood but leads to linear assembly. The current assembly model predicts that fibrillins, which are initially aligned head-to-tail, maturate to a one-third stagger alignment by folding at the termini and proline-rich regions. In tissues, assembled and matured microfibrils form loosely packed parallel bundles.

Several proteins such as fibronectin, α5β1 integrins and heparin sulphate proteoglycans are proposed to have a role in this assembly. As different extracellular microfibril populations have been identified, the extracellular environment might play a major role in regulating microfibril fate after assembly.

2/ **Tropoelastin** (TE), the precursor of elastin is a linear polypeptide of ~70 kDa, encoded by the elastin gene (*ELN,* chromosome 7q11.1-7q21.1) and is then secreted to the plasmamembrane via secretory vesicles. Its amino acid composition is rich in hydrophobic residues (Gly, Val, Pro, Ala), with glycine making out one-third of the total number of amino acids. Interactions between these hydrophobic domains play a crucial role in a process called coacervation. This process reflects the formation of aggregates of TE – which is soluble in a cold (< 20°C) aqueous solution – when temperature rises

towards the physiological range. Coacervation is an important step in fibrillogenesis of elastic fibres prior to crosslinking.
Next to hydrophobic domains, hydrophilic residues, including Lys and Ala, build up the crosslinking domains, allowing formation of covalent lysyl-derived desmosine crosslinks by an enzyme called lysyl oxidase [20].

Molecular events leading to assembly of microfibrils and TE into an elastic fibre are poorly understood, although several models have been proposed. It has been demonstrated that microfibril formation is followed by an accumulation of TE, gradually obscuring the microfibrils. This lead to the hypothesis that microfibrils form a scaffold on which TE molecules align and, throughout maturation, become predominant. As such, mircofibrils play a critical role in early fibrillogenesis, while the physiological properties of the elastic fibres are attributable to the matured elastin. Indeed, the alternating presence of hydrophobic and cross-link domains is likely to cause the elasticity of these fibres. When stretched, the hydrophobic domains are exposed to the surrounding aqueous milieu, and the energy for contraction of the fibres is derived from the return of these hydrophobic groups to the non-aqueous environment.

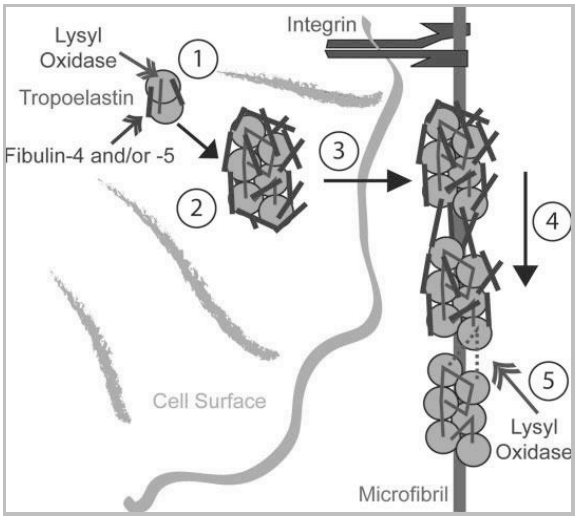

Figure 3
Schematic representation of microfibril and elastic fibre formation. (1) Transport and organization of tropoelastin into aggregates, cross-linked by LOX. (2) increase of aggregates size by newly secreted elastin. (3) Transfer of aggregates to extracellular microfibrils. (4) Elastin aggregates on the microfibril coalesce into larger structures. (5) Elastin aggregates are further cross-linked by LOX to form the complete elastic fiber.
Adopted from Wagenseil et al.

The **distribution and concentration** of elastic fibres varies in different tissues, being the highest in the arterial vascular tissues and the lungs, both characterized by a vast need of elasticity for their function. In other tissues, such as skin or liver, the concentration of elastin is rather low. Also the **architecture** of elastic fibres is highly tissue specific, reflecting tissue-specific functions. In the medial layer of blood vessels, the fibres form concentric fenestrated lamellae separated by smooth muscle cell layers. In the lung, elastic fibres are thin and highly branched while in the dermis they have a variable diameter and are horizontally or perpendicularly arranged.

The metabolic turnover of elastin – with a continuous degradation and replacement by newly formed fibres – is remarkably slow compared to other proteins. In pathologic conditions however, degradation is rapidly increased. The process of degrading elastic fibres is executed by a set of elastolytic enzymes, of which elastase is best known. First discovered in the pancreas, elastolytic enzymes have been detected in various cell types including polymorphonuclear leukocytes, monocyte/macrophages and platelets.

To allow maintenance of an optimal level of functional elastic fibres in tissues, the pathways involved in fibrillogenesis and degradation of fibres must be carefully controlled. If the balance in the rate of any of these processes is disturbed, the steady-state level of the fibres could be altered, manifesting as a pathological disease.

1.2.1.2 Other constituents of the ECM

Besides the elastic fibres, several other proteins are known to have an important structural and/or functional role in the dynamic function of the connective tissues. These compounds are altered in PXE but have no known central role in its pathophysiology and will therefore be discussed only briefly.

a/ collagen fibres

Collagen is a major component of the extracellular matrix and the most abundant protein in the body, accounting for about 25 to 30% of the total protein mass. Collagens are triple helical proteins formed when three polypeptide chains, called alpha chains, wind around each other to form a collagen molecule. The alpha chains are rich in the amino acids proline and glycine, which are important for the helical conformation. Because of its ring structure, proline stabilizes the helix. Glycine, the smallest of the amino acids, is spaced at every third position such that it occupies the tightly spaced inside portions of the triple helix [21].

Posttranslational modifications, including hydroxylation of specific proline and lysine residues, occur during the processing of the procollagen molecules and are important in stabilizing the collagen structure and in forming interchain and intrachain cross-links [21].

The collagens can be divided into different groups by structure and function. Fibrillar collagen molecules (collagen types I, II, III, V, and XI) are packed in a staggered and ordered fashion to form a collagen fibril, which in turn will be packed together to form fibres. Fibril formation occurs outside the cell after the N-terminal and C-terminal propeptides present on procollagen molecules are proteolytically removed to form mature collagen. Further cross-linking of collagen fibrils strengthens the structure. These rather stiff, rope-like structures provide the tensile strength for connective tissues. The fibril-associated collagens (collagen types IX, XII, XIV) help to form and stabilize the collagen fibrils. Unlike the fibrillar collagens, this group of collagens contains regions where the triple helical structure is interrupted, which can result in a bend in the collagen molecule, thus the name *fibril-associated collagens with interrupted triple helixes*. Type IV collagen is the classic basement-membrane collagen that forms in aggregates to provide structural support to the basement membrane [21].

b/ proteoglycans

Proteoglycans are build out of a protein core to which short oligosaccharides and longer chains of glycosaminoglycans (GAGs) are covalently attached. There is a large variety of proteoglycans based on the types and lengths of GAGs as well as the sequence and length of the protein core. Chondroitin sulfate, keratan sulfate, heparin sulfate, and dermatan sulfate are four different forms of GAGs that consist of repeating disaccharide units. The sulfates present on the GAGs create a highly negatively charged environment that is hydrophilic. Thus, connective tissues that contain large amounts of proteoglycans will also contain relatively large amounts of water bound to the proteoglycans. In addition to water, proteoglycans bind cationic proteins, which in some cases include growth factors, resulting in a mechanism by which tissues can store growth factors in the matrix.

The size of these proteoglycans can vary significantly. Aggrecan, predominantly found in cartilage is a very large protein (molecular weight of more than 200,000 kDa), while decorin, biglycan, fibromodulin, and lumican are small proteoglycans (30 to 60 kDa). The latter interact with collagen and function to regulate the formation of collagen fibrils and help stabilize the collagen network. Although their functions are not completely understood, they likely have other roles based on their ability to bind other matrix molecules such as elastin and fibronectin and on their affinity for growth factors.

c/ glycoproteins

Along with the proteoglycans, the many other noncollagenous **matrix glycoproteins** found in connective tissues form much of what is sometimes described in histological terms as the "ground substance". This rather inert-sounding description should in no way be taken to suggest that these matrix components simply fill in or hold the tissue together. Rather these proteins participate actively in creating the information-rich environment described earlier. Proteins in this group include fibronectin, vitronectin, osteopontin, laminin, and thrombospondin. Fibronectin is found in most connective tissues throughout the body. Laminin is particularly prominent in basement membranes, and osteopontin is found in greater amounts in cartilage and bone. All of these proteins bind to cells through specific cell-surface receptors to promote attachment of cells to the matrix. The proteins also interact with the other matrix proteins to further integrate the cells with the extracellular matrix. Through these interactions, they function in regulating tissue morphogenesis as well as tissue repair and remodelling. They also appear to play a role in other diverse processes, including tumour growth and metastasis.

1.2.2 Connective tissue disorders (CTD)

Heritable disorders that involve connective tissue are among the most common genetic diseases in humans. The first comprehensive effort to classify these diseases was made by Victor McKusick in a series of reports and then in the monograph *Heritable Disorders of Connective Tissue.* Based on pattern of inheritance, the cluster of signs and symptoms, the histological changes in tissues and limited information about the molecular defects involved, he drew the first classification of CTD. In the advent of molecular genetics, some limitations of

McKusick's categories became apparent. One is the phenotypic variability within and between families which characterises several of these disorders. Classification also tends to overemphasize the etiologic differences between severe genetic disorders that are apparent in infants or young children and the more common diseases that appear later in life. Yet these late-onset diseases, such as osteoporosis, aneurysms, osteoarthritis and stroke can be caused or influenced by single-gene variants. The debate on whether these patients should be classified as having a mild form of the CTD which is linked to the defective gene remains ongoing and will probably never be solved. These individuals have however made it clear that the class of "disorders of the connective tissue" needs to be expanded.

Because of the wide distribution of connective tissues within the human body, diseases that affect connective tissue cells or extracellular matrix proteins often have systemic effects. Based on the two major constituents of the connective tissue, collagen and elastin, these disorders can be divided into '**collagenopathies'** and '**elastinopathies'**. The most common collagenopathies include the a.o. Ehlers-Danlos syndromes, osteogenesis imperfecta, Alport syndrome and the chondrodysplasias. Elastinopathies include a.o. Marfan syndrome and the cutis laxa syndromes. However, one of the most intriguing examples of an elastinopathy – although this label does not meet the complexity of the disease –, is pseudoxanthoma elasticum.

1.3 Pseudoxanthoma elasticum

Pseudoxanthoma elasticum occupies a unique position within the connective tissue disorders, because of its molecular and etiopathological characteristics. PXE is found in all populations, with a higher prevalence in the Afrikaner population of South Africa due to a founder effect [22, 23]. Its world-wide prevalence has been estimated at 1:25000, with a predominance of female patients (female to male ratio of 2:1) when ascertainment from the skin changes is made [23, 24]. Although it has been previously suggested that there may be an estrogen effect, based on the aggravation of cardinal features during pregnancy, it is far more probable that sex predilection may be due to cosmetic concerns [25].

A **classification of PXE** into clinical subtypes has been previously proposed by Pope et al. and revised by Lebwohl et al. [26-28] Pope, in 1974, suggested that two autosomal recessive and two dominant forms existed, describing several clinical differences between them [26, 27]. In the years to come, several authors refuted this initial classification as it became clear that most – if not all - cases were autosomal recessive with a marked variability in expression [22, 29-34]. The few pedigrees which were truly suggestive for autosomal dominant inheritance, were up until now all shown to be due to pseudodominance.

Recent findings have changed our perspective on the disorder from a strict CTD to a metabolic disease with secondary connective tissue implications [35]. A summary of the current knowledge is presented against a background of physiological facts, necessary for the comprehension of this thesis.

1.3.1 Pathology

The primary histological alteration in PXE is degeneration of elastic fibres that undergo progressive mineralization and fragmentation, resulting in a histological image called "**elastorrhexis**" (Figure 4) [36, 37]. These alterations can be observed through light and electron microscopy in the main organs affected by PXE (skin, retina, blood vessels), but also in other tissues which contain elastin. The latter include the urinary system (kidney, bladder), the gastro-intestinal tract (oesophagus, intestines) and the pulmonary system (trachea, lung) [36]. The alterations in these systems, although often widespread and associated with collagen abnormalities, are however usually very small which could account for their lack of clinical significance [36].

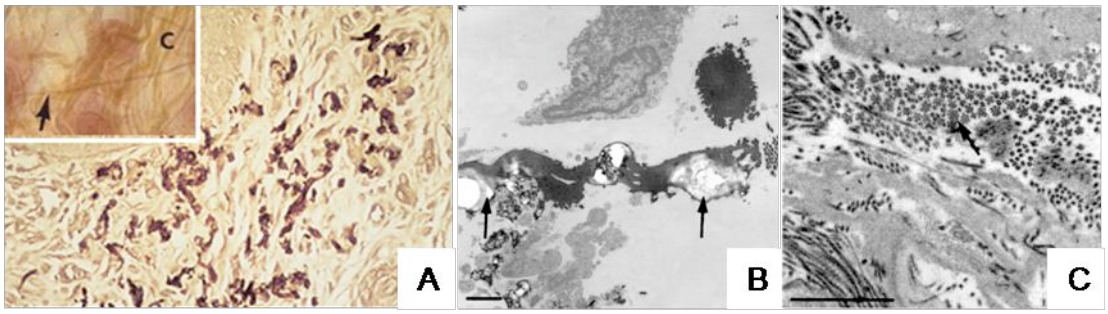

Figure 4

Histological alterations in PXE. Panel A shows the normal thin elastic fibres (insert) and a lightmicroscopical Von Kossa stain revealing elastorrhexis (x400). Panel B shows an electron microscopical image of calcifications (arrowed) throughout the core of an elastic fibre (Bar = 1µm). Panel C illustrates the presence of collagen flowers (arrowed) (Bar = 1µm). Panel B and C adopted from [36]

Although subtle but distinct differences can be noted in the histological findings in the different affected tissues, the **chain of degenerative events** is always alike. One of the intriguing questions in the chain of events – similar to the chicken or the egg dilemma –was whether the *primum movens* was the calcification or the fragmentation [38]. Both are theoretically possible as calcified fibres are prone to degradation, while fragmented fibres become more easily calcified. Several EM studies have however reported calcification in elastic fibres that appear normal as the first pathological sign in young individuals, allowing to put forward a pathological cascade as follows [38-40]. Initially, mineralization of the elastic fibre is seen as a central core of electron density on EM, with core density increasing as mineralization continues. Prior to fragmentation, the elastic fibres will develop holes, where the central portion of the core disappears or spontaneously fades. Finally, the fibres become maximally calcified, followed by fragmentation.

Two main kinds of calcification have been described: one composed of hydroxyapatite and the other of $CaHPO_4$ [41]. Other mineral precipitates, such as iron, phosphate and carbonate have also been identified in altered elastic PXE fibres [42-45].

A series of studies have described abnormalities of other ECM components, such as collagens and proteoglycans. **Collagen flowers**, a sign of abnormal collagen fibrillogenesis, are – although commonly found in PXE – also highly aspecific. Abnormal amounts of **proteoglycans** are localized nearby and within mineralized elastic fibres and abnormal amounts of GAGs, as well as alterations in their synthesis and deposition have been detected in PXE patients [46-49]. Moreover, PXE cells have been shown to produce proteoglycan species with altered properties,

such as stronger polyanion properties, increased hydrodynamic size, abnormal hydrophobic actions and different content and distribution of heparan sulphate, indicating an abnormal proteoglycan metabolism [49, 50]. In urine of PXE patients and carriers, both decreased and increased concentration of GAGs has been observed [51, 52]. Although no straightforward explanation exists for these discrepant findings, Maccari et al. found three distinguishing differences of urinary GAGs in PXE: the chondroitin sulphate/heparin sulphate ratio (which is lower), the 4-sulphated/6-sulphated chrondroitin sulphate ratio (which is lower) and the high degree of chrondroitin sulphate sulfation [52].

Baccarani-Contri et al. showed that elastic fibres have enhanced expression of normal constitutive proteins (e.g. vitronectine), but also accumulated aberrant matrix proteins known for their high affinity for calcium and involvement in mineralisation processes (e.g. alkaline phosphatase, bone sialoprotein, osteonectin) [48].

Several **histological stains** can be applied to reveal the mentioned pathological features of PXE. Aberrant clumped and fragmented elastic fibres are demonstrated by hematoxylin-eosin-safran staining or by the use of specific elastin stains (orcein, Verhoeff-Van Giesson). Ectopic mineralization can be shown by a Von Kossa stain, which colours calcium black. The presence of proteoglycans in the vicinity of the increased amount of abnormal elastin fibres can be demonstrated by alcian blue or colloidal iron stain.

1.3.1.1 Calcium homeostasis

Physiological mineralization is a crucial metabolic function which is restricted to specific sites in skeletal tissues, including growth plate cartilage, bones and teeth. Uncontrolled or pathological calcification can occur in every tissue, although articular cartilage, cardiovascular tissues and the kidney are particularly prone. The regulation of physiological mineralization encompasses a complex network of metabolic pathways. Being cell-mediated, it involves the coordinated expression of and interactions between stimulatory and inhibitory factors.

a/ matrix vesicle-mediated mineralization

Matrix vesicles initiate the mineralization process in growth plate cartilage and bone. These vesicles are released from the plasma membrane of mineralization-competent cells into the ECM. Channel proteins mediate the influx of calcium ions and inorganic phosphate into the vesicles, in which a calcium-inorganic phosphate-phospholipid complex is formed when being released from the plasma membrane. This complex serves as a nucleus for the formation of the first intravesicular mineral phase. Once the mineral has reached a certain size, it ruptures the vesicle membrane and grows into the ECM [53].

b/ mineralization propagators and inhibitors

To propagate extravesicular crystal growth, an appropriate “calcifiable” matrix needs to be present. It has been shown that both collagen type I, II and X are required for ectopic mineralization. Interaction of type II and type X collagen with annexin V, a channel protein for calcium, leads to a rapid influx of calcium into the vesicle [54].

A second important protein group implicated in regulation of mineralization are the **Gla-proteins** (Figure 5). Named after the glutamic acid (gla) residues which they contain, they all have a similar metabolic processing comprising transcription and translation to an inactive protein, followed by post-translational modification. This post-translational modification consists of the carboxylation of one or more gla-residues by a gamma-carboxylase enzyme.

The **γ-carboxylase** (GGCX) is one of two enzymes within the vitamin K-cycle, together with the vitamin K epoxide reductase (VKOR; Figure 5). For these enzymatic processes, vitamin K acts as an essential co-factor, which needs recycling due to the limited amount of vitamin K in the diet. Several vitamin K-dependent (or Gla-) proteins have been discovered, which undergo processing via this metabolic cycle. These include **coagulation factors** II (prothrombin), VII, IX and X, protein S and protein C but also several **inhibitors of calcification**, such as matrix gla protein (MGP) and osteocalcin (OC), and four integral membrane proteins of unknown function. The carboxylase enzyme itself is negatively regulated by a protein called calumenin.

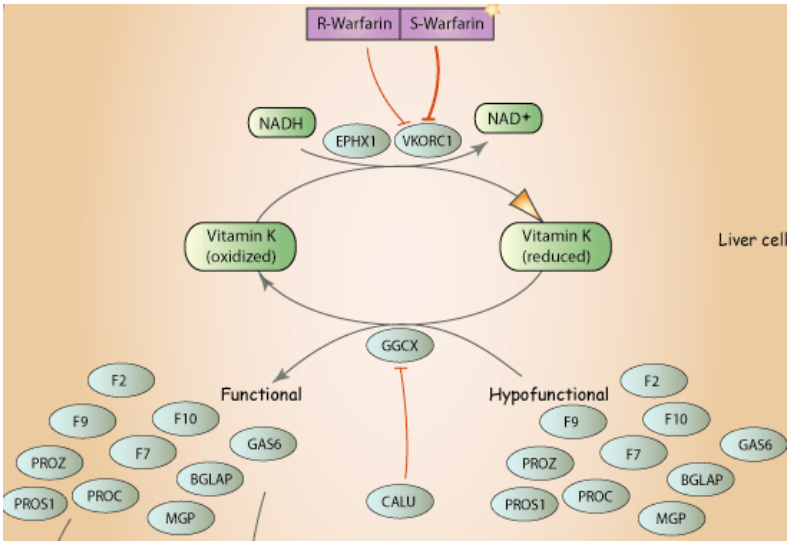

Figure 5

The vitamin K cycle. Reduced vitamin K acts as a co-factor of the γ-carboxylase GGCX, activating hypofunctional proteins. The vitamin K, which becomes oxidized during this modification, is reduced by VKOR.
F2-7-9-10: vitamin K dependent clotting factors; PROC: protein C; PROS: protein S; PROZ: protein Z; MGP: matrix gla protein; BGLAP: bone gla protein (or osteocalcin); GAS6: growth arrest-specific gene 6; CALU: calumenin.

MGP is expressed in vascular smooth muscle cells and chondrocytes but not in osteoblasts, whereas OC is expressed in osteoblasts, odontoblasts and terminally differentiated chondrocytes. Whereas OC-deficient mice show no alteration in ECM mineralization, *Mgp-/-* mice show extensive vascular and cartilaginous mineralization, leading to early death due to arterial rupture [55, 56]. The human correlate is **Keutel syndrome**, an autosomal dominant disorder characterized by extensive cartilage calcifications which, in contrast to the animal model, is however not lethal [57]. The presence of MGP has been demonstrated in association with the ECM and specifically the elastic laminae in the human arterial vessel wall [58]. In areas of vascular calcification, colocalization of MGP and the elastic laminae is lost and MGP is found at the borders of vascular mineralization. It has been shown that absence of MGP promotes atherosclerosis and plays an important role in ectopic calcification seen in terminal renal insufficiency patients in need of dialysis [59, 60].

Other negative regulators of uncontrolled mineralization are **osteopontin** (OPN) and **pyrophosphate**. OPN, a phosphoprotein expressed in the growth plate cartilage and bone, can act as in inhibitor or activator of mineralization, depending on the phosphorylation degree [61]. Recently, it has been shown that OPN is upregulated in Mgp-/- mice, pointing towards a possible secondary effect of absent MGP. Pyrophosphate is an inhibitory protein which itself is regulated by interactions of glycoprotein-1 – generating pyrophosphate –, ANK – a cell-membrane associated protein transporting pyrophospate to the ECM –, and TNAP – hydrolysing organic phosphor compounds – although the latter may be particularly important in removing the inhibitor from the mineralization site [62]. The *Ank-/-* mouse, similarly to *Mgp-/-* mice, features extensive soft tissue mineralization [62].

Fetuin A (α_2-Heremans-Schmidt glycoprotein) is a systemic calcification inhibitor synthesized by the liver. The fetuin A deficient mice (*Ahsg-/-*) developed severe calcification of various organs, while calcium and phosphate homeostasis remained unchanged [63]. Further extensive genetic evidence for the inhibitory role of fetuin A was given by ultrastructural and biophysical data demonstrating that inhibition of calcium precipitation by fetuin A is caused by formation of soluble, colloidal spheres containing fetuin A, MGP, calcium and phosphate [64, 65]. These “calciprotein particles” are more soluble than calcium and phosphate alone but may become progressively more crystalline and insoluble in a time- and temperature-dependent manner.

Finally, the **BMP-pathway** has been implicated in mineralization homeostasis. Bone morphogenetic proteins or BMPs are a group of growth factors and cytokines known for their ability to induce formation of bone and cartilage [66]. In total, 16 BMPs have been described, six of which belong to the TGFβ-superfamily of proteins. BMPs interact with specific cell surface receptors, through a signalling pathway involving members of the SMAD protein family. Of the BMPs involved in mineralization, BMP-2 acts as a disulfide-linked homodimer, inducing bone and cartilage formation. It also has a crucial role in osteoblast differentiation, similar to BMP-7. In addition, BMP-3 and BMP-8a are involved in bone and/or cartilage development [66]. The BMP pathway is regulated by several activators and inhibitors. A well-known example of the latter is MGP, which has been shown to inhibit the function of BMP-2 [67].

1.3.1.2 Ectopic calcification

Deposition of calcium crystals or true bone formation in non-osseous soft tissue (or ectopic calcification/ossification) may occur by one of three mechanisms:

1/ **metastatic** calcification due to a supranormal calcium x phosphate concentration product in extracellular fluid

2/ **dystrophic** calcification due to mineral deposition into metabolically impaired or dead tissue despite normal serum levels of calcium and phosphate

3/ ectopic **ossification** or true bone formation

Disorders that may cause extraskeletal calcification or ossification are listed in table 2. Despite the abnormal calcium x phosphorus product, several disorders in which metastatic calcification occurs, such as renal failure and hemodialysis, have been shown to also present abnormalities of the physiological inhibitors of calcification, including MGP. As such, abnormal ionic serum levels probably do not fully account for the pathological features. A severe and aggressive disease is fibrodysplasia ossificans progressiva or FOP, in which a deficiency of the BMP pathway of mineralization inhibitors finally leads to widespread ectopic ossification, often leading to premature death due to respiratory failure. Because of the normal values of calcium and phosphate, the mineralization in PXE is defined as dystrophic calcification. In rare cases, ectopic ossification has been reported, as described further.

Metastatic calcification	Dystrophic calcification	Ectopic ossification
Primary hyperparathyroidism	Scleroderma	Myositis ossificans
Sarcoidosis	Dermatomyositis	Fibrodysplasia ossificans
Vitamin D intoxication	Systemic lupus erythematosus	progressiva
Milk-alkali syndrome	Pseudoxanthoma elasticum	
Tumoral calcinosis		
Secondary hyperparathyroidism		
Pseudohypoparathyroidism		
Renal failure		
Hemodialysis		
Tumor lysis syndrome		
Therapy with vitamin D & phosphate		

Table 2
Diseases and conditions associated with ectopic calcification and ossification

1.3.2 Clinical findings

PXE is characterized by a marked clinical variability, even between siblings [22-24]. While the organ systems affected – skin, eye and cardiovascular system – are the same in all patients, the age of onset, severity of organ system involvement and natural course display a remarkable variability.

1.3.2.1 Skin and mucosal membranes

a/ basic anatomy

The skin forms the continuous external surface or integument of the body and is as such considered to be our largest organ. Four types of skin can be recognized, based on the region of their occurrence, which all have a similar histological structure consisting of 3 main layers:

1/ the **epidermis**, the external surface of the skin consisting of a keratinised squamous epithelium. the epidermis is build out of 5 distinct layers reflecting the maturation of epidermal cells formed by mitosis in the inner or *germinal layer*.

2/ the **dermis**, a thick layer of dense, fibro-elastic tissue, supporting and nourishing the epidermis. This highly vascularised layer is divided into the superficial *papillary dermis* – a loose network of bloodvessels and fine interlacing collagen fibres – and the larger so-

called *reticular layer* of the derm, because of the reticular arrangement of the collagen fibres.

3/ the **hypodermis** or subcutaneous layer which contains variable amounts of adipose tissue.

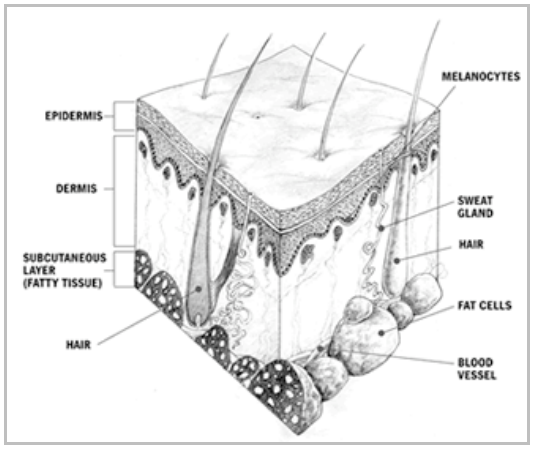

Figure 6
Schematic structure of the skin
Adopted from Virtual Science fair

Elastic fibres are important constituents of both layers of the dermis, forming a fine interlacing network of fibres in the papillary dermis, whereas in the reticular layer long thick fibres which follow the path of the coarse interlacing collagen bundles. The cells of the dermis are mainly fibroblasts, responsible for production of collagen, elastin and the ground substance.

b/ histopathology of the mid-dermis

Elastic fibres in the mid-dermis are typically polymorphous, mineralized and fragmented, while those in the papillary dermis and deep dermal layers have normal morphology. By EM**, two types of mineralization** can be noted: fine deposits in the center of the fibre and bulky precipitates deforming and breaking the fibres [36, 68, 69]. In these dermal mineralized areas, deposits of thread-like material, collagen flowers and collagen fibrils of irregular diameter are present in most patients. Fibroblasts are often numerous, with hypertrophy and dilatation of the endoplasmatic reticulum [36]. Near the mineralized areas, macrophages are abundant. Interestingly, ultrastructural elastic tissue alterations can be observed in both lesional and clinically non-involved skin, while the other ECM changes are only seen in clinically involved skin in vicinity of aberrant elastic fibres [23]. In rare cases, the dermal calcification can lead to ossification [70]. Interestingly, this has always been noted in those patients with significant cutis laxa. Of note, the dermal EM alterations are certainly not specific for PXE; they can also be seen in other inherited diseases of the ECM and normal skin aging [23]. Therefore, light microscopy evaluation remains essential for the diagnosis of PXE.

c/ clinical symptoms and evaluation

The skin features of PXE – resulting from clumping of the mineralized and fragmented elastic fibres in the mid-dermis – are characterized by a significant heterogeneity both in clinical severity and type of lesions. Although the skin manifestations are usually the first to appear, they are often not recognized as such, thus postponing the diagnosis [22]. During puberty, yellowish discoloured small 2-5 mm **papules** surrounded by normal skin can emerge, usually in the neck. Often referred to as cobblestone-like, Moroccan leather or plucked chicken skin texture, they are very characteristic for the disorder and, although they may enlarge and coalesce into larger **plaques**, they usually remain confined to the flexural areas of the body (neck, axillae, antecubital fossae, groins, popliteal spaces) and around the umbilicus [22, 23]. Although the face is ordinarily not markedly involved, Lebwohl et al. recently showed that horizontal and oblique mental creases have a high specificity for the diagnosis of PXE before the age of 30 [71].

In some patients, anogenital, gastric and mouth mucosae (most typically the inner lower lip) are affected, with a **yellowish reticular pattern** or – more rarely – small mucosal papules.

Figure 7

Skin features in PXE including papular lesions in the neck region and elbow (A-B-C), coalesced plaques of papules (D), cutaneous peau d'orange (E), increased axillary skin laxity (F) and mucosal involvement with yellowish reticular pattern of lower lip (G)

Long-term cutaneous changes may comprise loss of elasticity of the skin, resulting in redundant skin folds, particularly in the axillae. Some case reports described patients with a systemic cutis laxa-like PXE [70, 72, 73]. In rare cases, facial involvement can lead to a "hound-dog look". These long-term effects can be a significant esthetical and psychological burden. Only in very rare instances, spontaneous resolution of PXE skin changes has been noted; yet, in at least two cases it concerned acquired and hence pseudo-PXE [37, 74, 75].

More rare skin presentations which can be observed in PXE patients include Miescher elastoma [76, 77], elastofibroma [78], acne-like lesions on the neck or trunk, featuring comedones or inflammatory papules [79-81], elastosis perforans serpiginosa – a reactive perforating dermatosis characterized by elimination of abnormal elastic fibres from the upper dermis through

the epidermis – [82-89] a reticulate pigmented rash [90], or milia en plaque [91]. Keratoacanthomas – a benign cutaneous neoplasm – have been described located specifically on PXE skin lesions [92]. Bahadir et al. described irregular purple plaques in a patient, the histology of which was characteristic for PXE [70]. Nanda Kumar et al. reported a patient with multiple confluent atrophic plaques with peripheral hyperpigmentation, the histology of which was fully compatible with PXE [93]. Calcinosis cutis has rarely been reported in PXE patients but was always found in association with disorders of calcium and phosphate metabolism [94-97].

1.3.2.2 Eye symptoms

a/ basic anatomy

The human eye, although underrating its complexity, can be described as a cup-shaped photocamera (Figure 8). It is primarily designed to translate light into electrical impulses, which are then led to the visual cortex of the hindbrain through a complex network of intracranial visual pathways. For this purpose, light or images are projected onto the retina after appropriate focussing by the compound lens system formed by the cornea and the crystalline lens.

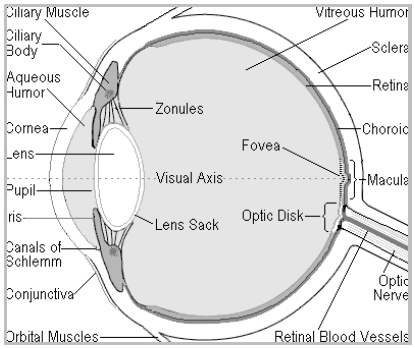

Figure 8
Schematic structure of the human eye
Adopted from Virtual Science Fair

The **retina**, approximately 500 micron thick, forms the innermost lining of the eyeball, and consists histologically of 10 layers, containing neural and/or supportive cells. It is bordered by the choroid, a highly vascularised layer and the sclera, the external coat of the eye. The most outer layer of the retina, a monolayer of cuboidal epithelial cells, is called the retinal pigment epithelium (RPE), which interacts with the photoreceptor layer of the retina and is separated from the choroid by **Bruch membrane** (BrM) (Figure 9). This elastin- and collagen-rich acellular membrane acts as an attachment site for the RPE cells and has a role in the vital bidirectional transport of oxygen and nutrients between the RPE and the **choroid**. Due to numerous interactions, the photoreceptors form one functional complex with the RPE cells and the ECM structure of BrM. This complex has a high level of metabolic activity and depends almost entirely on oxygen and nutrients derived from the choroid.

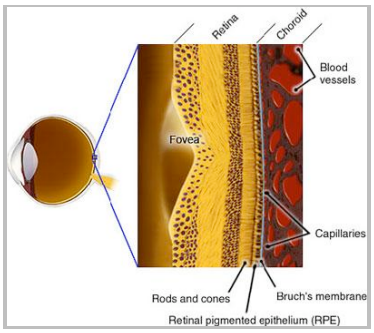

Figure 9

Transverse section through the posterior wall of the eye, showing BrM in between the retina and the bloodvessels of the choroid. Adopted from the American Health Assistance Foundation

Histologically, BrM consists of 5 layers:

1/ the **RPE basal lamina**

2/ the **inner collagenous zone**, consisting of collagens type I, III and VI

3/ the middle **elastic zone**, which forms a unique interlacing network with the inner and outer collagen fibrils, differing from elastin structures in the skin and bloodvessels. This maze-like structure is considered to play a role in the integration of the various ECM components into one functional unit.

4/ an **outer collagenous zone**

5/ the **basement membrane** of the endothelial cells of the choroid

In addition to the collagenous and elastic components, BrM contains a variety of proteoglycans (heparin, dermatan, chondroitin sulphate), which may be involved in the structural and functional properties of BrM, as they are suggested to make up anionic sites acting as charge barriers [98-100].

b/ histopathology of Bruch membrane

The changes in BrM are apparently identical to those seen in the middermis, with similar calcium deposits onto and clumping of fragmented elastic fibres. As a result, the barrier membrane will no longer have a smooth surface, but will start to exhibit breaks (Figure 10), making it possible for choroid vessels to grow towards the inner retina. The start of this degradation is characterized by discontinuities in the middle elastic layer and loss of RPE pigment granules. In a next stage, full thickness breaks in combination with atrophy of the overlying RPE and photoreceptor cells are observed plus ruptures of the underlying choroid. In some cases, herniation of choroidal fibrillar collagen tissue and choriocapillaris in to the breaks in BrM can be seen, separating this membrane. Surprisingly, no histological data on peau d'orange or comet tails (see paragraph 'd') could be found.

Figure 10
Graphic representation of BrM degradation (crack formation) and subsequent growth of bloodvessels through the breaks

c/ clinical evaluation

As light needs to be projected onto the retina, both the lens system of the eye as well as the vitreous liquid which fills the eye ball, are fully transparent. For this reason, the retina can be easily evaluated using a fundoscope (ophthalmoscope) and/or fundus camera.

Fundoscopically, the retina can be divided into the macula or yellow spot, of which the center or fovea is the region of highest visual acuity, and the retinal periphery responsible for the peripheral visual field (figure 11). The posterior pole, seen when the patient looks straight into the fundoscope, comprises the optic disk, macula and retinal midperiphery. In addition, a network of arteries, arterioles and veins can be seen in the superficial layers of the retina, supplying the inner cell layers of the retina.

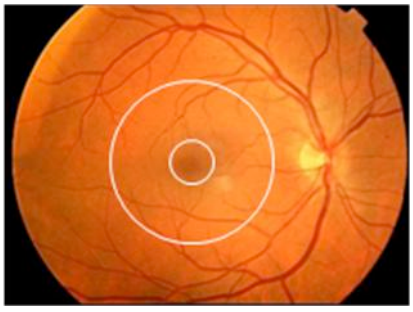

Figure 11
Normal fundus image of the right eye showing optic disc, macula (outer circle), fovea (inner circle), retinal midperiphery as well as retinal vasculature

Retinal **fundus photographs** will document the appearance of a retinopathy and serve as a baseline for future observations. In addition, retinal fluorescein and indocyanine green angiography may enhance recognition of retinopathy features [101-109].

Novel emerging fundus imaging techniques, such as autofluorescence, infra-red and red-free imaging – the mechanisms of which are explained in chapter 2 – have so far had no or limited use in PXE [110-113].

d/ clinical signs and symptoms

The five major components of the PXE retinopathy are (Figure 12):

1/ Ocular **peau d'orange (**Pd'O), a diffuse mottled hyperpigmentation of the fundus which is probably the first ocular sign of PXE [22, 23, 114, 115]. It consists of fine, relatively symmetrical flat lesions in the RPE. As it does not give rise to any symptoms, its exact time of origin is unknown and discovery is often postponed to a later age. Located in the retinal periphery, most prominent temporal to the fovea, approximately 28 to 73% of patients are presumed to have this aberration [116, 117]. The peau d'orange is however known to regress with age, significantly reducing the prevalence in elder PXE patients. When readily identifiable in one individual, siblings should be investigated for the presence of Pd'O, even in the absence of angioid streaks.

2/ **Angioid streaks** (AS) represent the breaks in Bruch membrane due to elastorrhexis. In fundo, they are seen as irregular, often jagged pigmented red-brown lines, radiating out from the optic disk, resembling normal retinal or choroidal vasculature at first glance, hence their name [25]. Sometimes, connecting streaks encircling the optic disk can be observed. AS are typically bilateral and variable in their number and extent. While in many patients there is a slow increase in number and length/width of streaks, they are asymptomatic and, although their mean age of onset is in the second decade, detection often only occurs when they are complicated by retinal haemorrhages. It is considered that all PXE patients will develop AS sooner or later and, if taken together with the other characteristics of the retinopathy, they have a high specificity for PXE [24, 25]. Occasionally, AS are found in isolation, but they have also been seen in younger individuals with PXE who had no cutaneous manifestations of the disease but developed them later. Therefore, when AS are identified in a patient, a systemic disorder as PXE must always be suspected and the patient should remain under surveillance.

3/ **Peripapillary atrophy**, can be observed in different forms (varying from helicoid to cuboid) and can be so extensive around the disk and in the macula that AS are no longer visible [24]. Generalized atrophy of the RPE and choroid, as well as reactive hyperplasia of the RPE with less marked AS have been described [23, 118].

4/ **Subretinal neovascularisation**, **haemorrhages** and **macular degeneration**, are regarded as complications of AS and were, in the past, often the first sign leading to the diagnosis of PXE. Neovascularisation engages from the choroid through the breaks in BrM, possibly triggered by Vascular Endothelial Growth Factor (VEGF). The novel vessel will push against the outer retinal layers, deforming the otherwise tightly stretched retina. Due to a lack of tight junctions in the neovessels, an exsudative reaction – leakage through the vessel wall – occurs. As a result, patients can experience metamorphopsia, a distortion of their vision, with straight lines becoming curved. As these neovessels also have brittle walls, rupture – either spontaneous or traumatic – can occur easily, resulting in a haemorrhage and subsequent (partial) vision loss (scotoma), depending on the size and location of the haemorrhage in fundo. When the macula is affected, patients lose

their central sight. It is however unusual for PXE patients to become completely blind; in most cases, peripheral vision is retained.
Subsequent neovascularisations and haemorrhages may lead to macular degeneration (so-called disciform process), the irreversible end-stage of the PXE retinopathy.

5/ **Comets** and/or **comet tails** are nodular white punched-out fundus lesions, representing crystalline bodies in the retinal midperiphery and juxta-papillary area with a variable degree of retinal pigment epithelium (RPE) atrophy. These white dots may extend to the sclera with a slightly depigmented tail in the RPE (hence comet tail). Gass, who coined the term comets, described these lesions as being the only fundus feature pathognomonic for PXE [119]. When found in isolated cases, they must be differentiated from the lesions seen in presumed ocular histoplasmosis (POHS), a fungus infection in which white dot lesions are however brighter and lack a tail [24] .

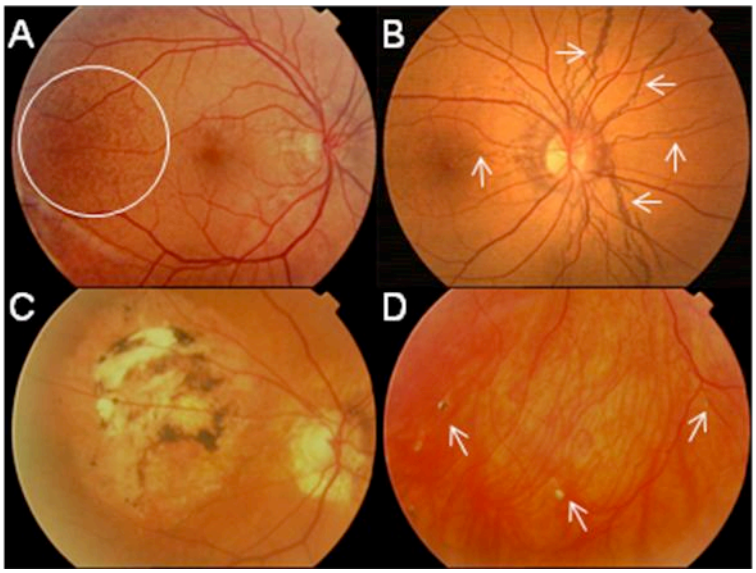

Figure 12
PXE retinopathy features with peau d'orange (A, circle) , angioid streaks (B , arrows), retinal haemorrhage and macular degeneration (C) and comets (D, arrows)

Other funduscopical findings include **drusen** of the optic nerve head (Figure 13) [120, 121], salmon patches (which typically represent a healing subretinal haemorrhage) [115], "black dots" (as a result of metaplasia of the RPE following focal haemorrhage) and chorioretinal arteriovenous communications [122]. Although the latter occur with significant higher prevalence in PXE, these features are less specific for the disorder. Historical references to chorioiditis or chorioretinitis in PXE are probably based on misinterpretations of the disciform process. Similarly, early case reports of blue sclerae and vulnerability to global rupture represent probably misdiagnosis of osteogenesis imperfecta [123].

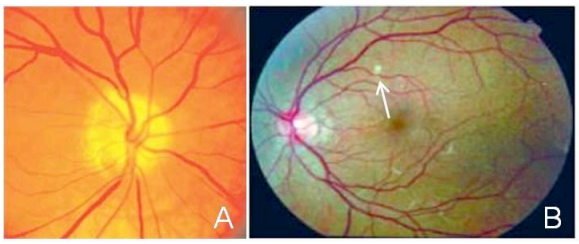

Figure 13
Fundoscopic image of optic disc drusen (A) and salmon spot (B, arrowed)

1.3.2.3 Cardiovascular symptoms

a/ basic anatomy

The cardiovascular manifestations of PXE are due to degradation of elastic fibres in the endocardium and arteries. ECM alterations in the latter are also responsible for the gastro-intestinal symptoms [124].

Histologically, arteries are divided up based on their location (peripheral or central arteries), size (small, medium-sized or large) or main histological component (elastic, muscular or musculo-elastic). In PXE, mostly peripheral, medium-sized muscular vessels are affected. The wall of these bloodvessel consists out of three layers:

1/ the tunica **adventitia**, an outer layer of connective tissue, supporting the nervi and vasa vasorum

2/ the tunica **media**, a middle layer of smooth muscle cells

3/ the tunica **intima**, made up of a single layer of endothelial cells and underlying connective tissue

These primary layers are separated from each other by two thin membranes which consist of elastic fibres and are therefore called the internal (between intima and media) and external (between media and adventitia) elastic laminae.

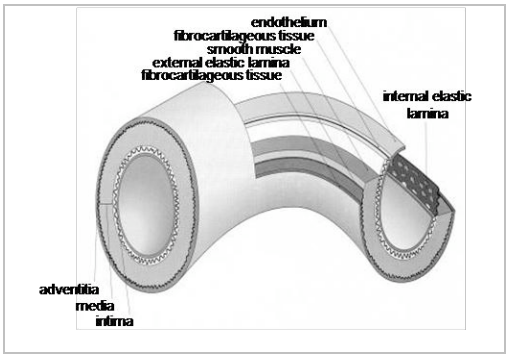

Figure 14
Transverse and horizontal section of a bloodvessel
Adopted from Stevens and Lowe, Basic Histology

The histological structure of the heart is very similar to that of bloodvessels, albeit that the terminology is different. Three basic layers can be distinguished:

1/ the **endocardium** – the innermost layer of the heart – is the equivalent of the tunica intima and consists of a single lining of endothelial cells and its supportive, collagenous tissue. Beneath the collagen lays a robust fibro-elastic layer, allowing movement of the myocardium without damage to the endothelium.

2/ the tunica media of the heart is called the **myocardium** and is made up of cardiac muscle.

3/ the **epicardium**, the tunica adventitia of the heart, consists of a single cell layer, the mesothelium, which is supported by a thin fibro-elastic layer.

b/ histopathology

Both the myocardium and pericardium show elastic fibre mineralization and collagen flowers, resembling the mid-dermal alterations [36]. Similar findings can be observed in nearly all small and medium-sized bloodvessels, including intestinal vessels, although it has been observed that alterations are not homogeneously distributed in the vessel wall. Alterations are most prominent close to the adventitia [36].

In contrast to the dermis and retina, vascular elastic fibres tend to form **aggregates** of thin strands of amorphous elastin which replaces the internal elastic lamina, e.g. in the aorta. These degenerative changes may be accompanied by various degrees of intima thickening due to a patchy proliferation pattern of the fibroelastic components [125]. These vascular changes show great comparison with Mönckeberg-type arteriosclerosis and the idiopathic type of generalized arterial calcification in infancy (IACA). The former is a type of focal calcific arteriosclerosis in the elderly, while IACA is a dramatic variant of arterial myoelastic degenerative tissue calcifications, caused by mutations in the *ENPP* gene and leading to intrauterine death or lethality within the first months of life.

Also the venous system, more specifically the vena cava, can be affected at the ultrastructural level in PXE [36].

c/ clinical signs and symptoms

Vascular morphological changes result in peripheral (PAD) and coronary (CAD) artery disease of various severity. Microangiopathy has been illustrated by capillaroscopy but deemed aspecific [126]. The most frequent symptoms and signs of PAD include:

1/ **hypertension** which has been reported in 8 to 22% of PXE patients; in some childhood cases, hypertension was the presenting manifestation of the disorder [125, 127-136].

2/ **intermittent claudication (IC)** of the lower extremities is described as the most frequent vascular complaint, occurring in ~30% of patients [137, 138]. Usually starting from the third decade on, it has been described in childhood in rare cases. Absence of

peripheral pulsations should be seen as an alarm symptom. Although in literature the term “accelerated atherosclerosis” is mentioned, the peripheral artery calcifications are usually patchy so that the resulting ischemia progresses slow enough to allow for the development of collateral circulation. As such, severe complications of IC, such as gangrene and total loss of an extremity are rare.

3/ **abdominal angina** resulting from celiac artery stenosis is, next to gastro-intestinal bleeding, the most common abdominal complaint in patients [77, 130].

4/ **stroke** has traditionally been considered a rare complication of PXE and mostly due to small vessel disease [139]. Childhood cases have been described [140]. Anecdotal case reports describe involvement of the cerebral or basilar arteries [93]. An association of PXE with intracranial aneurysms has been deemed unlikely, although no large case series are available to support this hypothesis [139].

5/ **increased compressibility of the arterial wall**, particularly of the carotid arteries, has been demonstrated in PXE patients, possibly because of the observed accumulation of proteoglycans in vessel walls [141, 142]. Large proteoglycans are considered key components for maintaining shape and sustaining compression generated by pulsatile forces and, by interacting with macromolecules entering the vascular wall (such as oxidized LDL), may favour atherosclerosis [143].

Cardiac involvement is uncommon in PXE, although a higher prevalence of diastolic dysfunction has been reported [144]. Previously described associations with valvular disease, in particular mitral valve prolapse, remain to be determined using stringent diagnostic criteria [145-148]. Restrictive cardiomyopathy in relation to diffuse endocardial fibroelastosis seems to be very specific for PXE but is also very rare [149]. Most common CAD manifestations in PXE patients are:

1/ **angina pectoris** or silent coronary insufficiency [150], which in two large clinical series occurred in 13 to 53% of patients [6, 37].

2/ **myocardial infarction** has not been reported frequently in association with PXE, although examples of very young patients are at hand [135, 151-158]. Thus, PXE should be considered in young individuals with precocious coronary artery disease and no cardiovascular risk factors[159].

Sudden death has only been described in four cases and may be due to fatal arrhythmias subsequent to large or small vessel coronary stenosis with ischaemia, conducting system and endocardial disruption [160, 161].

A serious vascular complication of PXE is **gastro-intestinal haemorrhage**, usually originating from the stomach, which has been reported in 8 to 19% of patients, and probably results from similar morphological changes in the submucosal gastric arteries as in peripheral arteries which – in contrast to PAD - lead to arterial fragility and haemorrhage [162-164]. Some authors have proposed that bleeding may be related to defective submucosal vasoconstriction [165]. Other etiological factors, such as increased gastric acidity with mucosal erosions and

damage of submucosal arteries have been suggested to play a role, although this has never been proven.

1.3.2.4 Other clinical manifestations

Although no patients with respiratory complaints have been described, **pulmonary opacities** in the lungparenchyma have been described in rare cases [166-169]. As the lung is very rich in elastic fibres, it remains unclear why this organ seems protected from clinically significant ECM degeneration.

Calcifications can be observed on **mammograms** in female PXE patients, which might be of diagnostic value in women, as breast tumour calcifications are usually easily ruled out [170, 171]. One case report demonstrated similar ectopic **calcifications in the testicles** of a 14-year old patient [172].

Several **auto-immune diseases**, including chronic cutaneous lupus erythematosus [173], rheumatoid arthritis and Hashimoto's thyroiditis have been described in association with PXE [174-179]. In most – if not all – cases hitherto reported, it is most likely to concern coincidental findings rather than a true pathogenetic association.

Most women with PXE have normal **pregnancies and deliveries** [180]. In the past, case reports have undoubtfully overemphasized the risk for gastric bleeding during the pregnancy as well as complications such as chorio-amnionitis, intra-uterine growth retardation or thrombo-embolic events [181-192]. Although the placenta may show more necrotic changes and mineralization compared to normal women, these are unlikely to provoke growth restriction or miscarriage [193]. A recent large case series concluded that there is no basis for advising women with PXE to avoid becoming pregnant and that most pregnancies in PXE are without complications [180]. Most effects of pregnancy directly on PXE relate to aggravation of the skin lesions [188, 189, 191]. Epidural **anaesthesia** can be established if necessary [194, 195]. It has been suggested that ophthalmological monitoring is important during labour to prevent retinal haemorrhages, while some authors advise delivery through caesarean section to avoid visual complications [196].

1.3.2.5 Phenotype in heterozygous carriers

Individuals carrying one mutated allele of an autosomal recessive disorder have traditionally been considered as “healthy carriers”, which do not develop any disease manifestations. In several disorders, this has been proven incorrect, as carriers were shown to have at least a partial phenotype. In PXE, the issue on whether or not the carriers of one *ABCC6* mutation have a clinically relevant (partial) phenotype has long been ongoing. It is certainly true that one *ABCC6* mutation is sufficient to **modify dermal elastic fibres** and – by extrapolation – fibres in BrM and blood vessels [197, 198]. Martin et al. identified three histological phenotypes of the skin elastic fibre system depending on the *ABCC6* genotype (patient vs. carrier vs. wild type), in which severity of disruption in carriers was between patients and unaffected individuals with short, thick and sometimes fragmented fibres, staining mildly for bone sialoprotein compared to patients [198].

In addition to the histological aberrations, several authors described one or more clinical manifestations in heterozygotes. Sherer et al. have reported on mild ophthalmological and/or cutaneous involvement in heterozygous carriers, but they did not indicate the frequency of this

phenomenon in their cohort of patients [199]. Additionally, no other reports of oculocutaneous involvement in carriers have been issued. More convincing are the reports emphasizing the carriage of one *ABCC6* mutation to be a **risk factor for cardiovascular disease**; the p.R1141X mutation has, in heterozygous state, been observed as an independent risk factor for coronary heart disease in young people [200, 201]. It seems probable that the course of disease in carriers is different – probably more beneficial – than in PXE patients. However, these findings emphasize the importance of screening other – apparently healthy – individuals in a PXE family for carriership of an *ABCC6* mutation. Depending on their confirmation in other cohorts, these observations may represent considerable concerns for PXE families and public health in general.

1.3.3 Molecular genetics

The molecular era for PXE was launched in 2001 – four years after the first mapping of a chromosomal region on 16p13.1 – with the identification of causal mutations in the *ABCC6* gene [12, 14, 202]. Prior to its characterization, the initial 16p locus was refined to a region of about 500 kb, containing 5 genes (*ABCC1*, *ABCC6*, *pM5*, and two copies of the *NPIP* gene) [9, 11, 203], direct sequencing of which identified the pathogenic gene. The involvement of *ABCC6* was unexpected, as its protein, an ATP-dependent transporter, had no apparent relation with ECM proteins or homeostasis. Although our understanding of the domain structure has increased significantly, *ABCC6* still harbours several unclarities and apparent contradictions.

1.3.3.1 The ABC transporter superfamily

The ATP-binding cassette (ABC) superfamily involves 48 human functionally diverse active **transmembrane transporters**. Strongly conserved in different species, they play a fundamental role in the inter- and intracellular membrane transport of a.o. amino acids, lipids, lipopolysaccharids, anorganic ions, peptides, saccharids, metals, drugs and proteins. They are implicated in signal transduction, drug resistance, protein secretion and antigen production. Although most often directly involved in substrate transport based on **ATP hydrolysis**, some appear to be part of ion channels where ATP hydrolysis regulates opening and closing. Most transporters function unidirectional, although for some members, bidirectional transport capacities have been described.

The typical ABC-transporter (so-called **full-transporter**) consists of two transmembrane domains (TMD), responsible for substrate specificity and containing 6-11 transmembranary helices, and two highly conserved hydrophilic nucleotide bindings folds (NBF), build up of two sequence motifs (Walker A & B) and a consensus sequence (C-motif), critical for ATP binding. **Half-transporters** have only one TM and NBF and need coupling to become active (Figure 15).

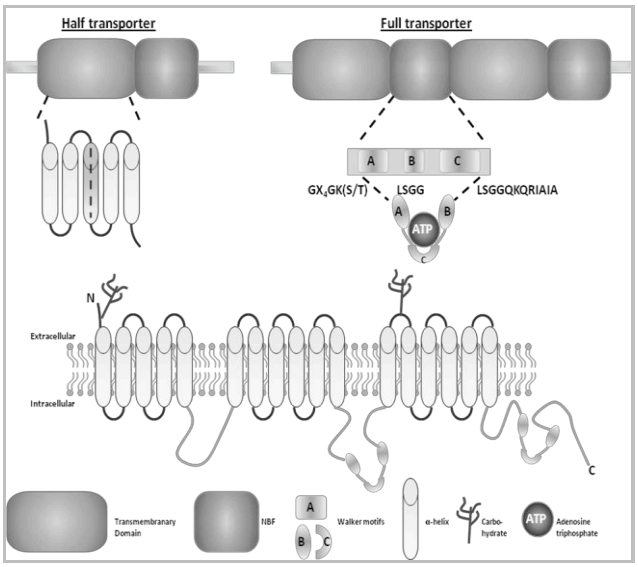

Figure 15
Characteristics of the human ABC transporters

ABC transporters are divided into **7 subclasses** (A through G), based on phylogenetic analysis. Fourteen out of the 48 transporters have been implicated in 13 genetic disorders, the most common of which are cystic fibrosis (ABCC7), Stargardt disease (ABCA4), adrenoleukodystrophy and Tangier disease. The ABCC subfamily contains 12 full transporters with a diverse functional spectrum (Table 3).

Transporter name	Function	Disease	OMIM#
ABCC1 (MRP1)	Drug resistance	Unknown	
ABCC2 (CMOAT)	Bile acid transport	Dubin-Johnson syndrome	237500
ABCC3	Drug resistance	Unknown	
ABCC4	Nucleoside transport	Unknown	
ABCC5	Drug resistance	Unknown	
ABCC6	Unknown	Pseudoxanthoma elasticum	264800
ABCC7 (CFTR)	Chloride ion channel	Cystic fibrosis	219700
ABCC8	Sulfonylurea receptor	FPHH, DM type II	256450/600509
ABCC9	K(ATP) channel regulator	Dilated CMP with VT	608569
ABCC10	Unknown	Unknown	
ABCC11	Unknown	PKC*	128200
ABCC12	Unknown	ICPC*	602066

Table 3
Members of the ABCC subfamily, their function and associated diseases
(*) Candidate gene; FPHH: Familial Persistent Hyperinsulinemic Hypoglycemia (nesidioblastosis), DM: Diabetes Mellitus; CMP: Cardiomyopathy; VT: Ventricular Tachycardia; PKC: Paroxysmal Kinesigenic Choreoathetosis; ICPC: Infantile Convulsions with Paroxysmal Choreoathetosis
Adopted from Stefkova et al. [203]

With the ABCC6 transporter, previously referred to as MRP6 (multidrug resistance protein 6), implicated in PXE, a fairly unknown member of the ABC superfamily came to attention. It is composed of three hydrophobic membrane segments comprising five, six and six transmembrane spanning domains, respectively, and two nucleotide bindings folds (Figure 15). ABCC6 was classified as an MRP, because of its homology with MRP1, reaching 45% identity. While MRP1 is a well characterized transporter of amphipathic anionic conjugates, glucuronidated and sulphated compounds, the transport capacities of ABCC6 remain unclear [205].

In vitro, ABCC6 was demonstrated to have ATP-dependent transport capacities of at least two anionic glutathione conjugates (leukotriene C, N-ethyl maleimide S-glutathione [NEM-

SG]), comparable to ABCC1 and 2 [206, 207]; organic anions known to interfere with glutathione conjugate transport inhibited NEM-SG in a specific manner, indicating ABCC6 to have a unique substrate specificity. It also confers low levels of resistance to several agents such as etoposide, teniposide, doxorubicine and daunorubicin [206]. The affinity of ABCC6 for these compounds is however very low, excluding them as plausible physiological substrates.

Using several polyclonal antibodies, ABCC6 was localised to the basolateral side of human **hepatocytes** and to the basolateral membrane of **kidney proximal tubules** [208]. This subcellular localisation suggests that the transporter **extrudes** into the blood specific substrates from liver and kidney. However, the search for the genuine physiological transport substrate of ABCC6 and hence its **physiological function** currently still continues.

1.3.3.2 The *ABCC6* gene

The *ABCC6* gene, located on chromosome 16p13.1, comprises 31 exons spanning ~73 kb of genomic DNA. The transcribed messenger RNA is 6 kb, with a coding region of ~4.5 kb, leading to translation of a protein of 1503 aminoacids [14].

Closely mapped to *ABCC6*, two **pseudogenes** (*ABCC6-φ1* and *ABCC6-φ2*) were identified with high homology of the promoter region as well as exon 1 through 9 and exon 1 through 4 respectively [209].

Surprisingly, the highest *ABCC6* expression is observed in the kidneys and liver, while tissues involved in PXE (skin, retina, blood vessels) only revealed low expression levels [12, 206, 210]. A series of other tissues – including brain, tongue, stomach and small intestine – demonstrate very low levels of *ABCC6* expression [211, 212]. Based on this remarkable expression profile and the subcellular localization of the ABCC6 protein, PXE has recently been considered a **metabolic disease**, in which a so far unknown metabolite – or lack of it – is responsible for the connective tissue alterations.

The **regulation of *ABCC6* expression** and activity is not yet completely understood and – based on current knowledge – is deemed highly complex, involving regulatory proteins, DNA methylation determining tissue-specificity, liver and erythroid-specific enhancers and pro-inflammatory cytokines. The high hepatic expression suggested that transcriptional *cis*-elements confer high liver-specific expression of the gene. This was confirmed by promoter-reporter gene constructs in HepG2 cells [213]. Subsequently, a NF-κβ-like segment (the promoter segment – 249 and -176) was discovered, functioning as a liver-specific element which binds HepG2 cell nuclear proteins in a specific manner. Other sequences for liver-enriched transcription factors could not be detected, suggesting that this *cis*-element is responsible for the high hepatic expression of *ABCC6* [213]. The complexity of transcriptional regulation was further unravelled by the identification of at least four eukaryotic transcription factors (activator protein-2, USF-1, NF-κβ and epidermal growth factor) to the -2631 to +30 promotor region [213]. Arányi et al. described both activator and repressor sequences in the proximal *ABCC6* promotor region; the most potent activator sequence consisted of conserved elements protected by DNA methylation in non-expressing cells [214]. In murine *abcc6* gene regulation, the importance of the proximal promotor was also stressed by identifying HNF4α (Hepatocyte Nuclear Factor 4 alpha) and NF-E2 (Nuclear Factor Erythroid 2) as transcriptional regulators, enhancing the promotor activity of *abcc6*.

Recently, two members of the PLAG family of cell cycle progression-related DNA-binding proteins, PLAG 1 and PLAGL1, were identified as transcriptional regulators of the human *ABCC6*

gene [215]. This interaction is suggested to be direct and mediated by a single response element (PLAG binding site) in the *ABCC6* proximal promotor. The events occurring upstream from the PLAG proteins remain however unclear [215].

ABCC6 **promotor activity** can also be modulated by several cytokines, including transforming growth factor β (TGF-β), tumour necrosis factor α (TNF-α) and interferon γ (IFN-γ). In particular, TGF-β, a profibrotic cytokine from the liver, significantly upregulated promoter activity [213, 216]. For TGF- β responsiveness, an upstream Sp1 (Specificity protein 1) binding site at -58 to -49 was shown to be critical as was already suggested by other investigators for other genes [213, 217, 218].

Currently, more than 150 **mutations** have been described in nearly all exons of *ABCC6*, albeit with a predominance of mutations at the 3' end of the gene [12-15, 23, 33, 155, 197, 202, 219-239]. All types of mutations have been described, although the majority are missense. Of these, about half are located in exons 15 through 19 and 26 through 31, encoding the two NBF's, replacing critical aminoacids and thus projected to compromise ATP hydrolysis. Functional experiments on these missense mutations are scarce, but findings on three missense mutations in NBF2 confirm the hypothesis of abolished transport function [207]. As several missense variants are located in intracellular domains outside NBF 1 and 2, this suggests that other domains may be functionally important, e.g. for substrate recognition.

Although most mutations are confined to a limited number of families, **two recurrent mutations** have been described. The p.R1141X (c.3421C>T) nonsense mutation is the most frequent mutation in a European population, although frequencies differ between different countries [232]. For the South-African population as well as for the French and Italian populations, a founder effect was described for p.R1141X [229, 235, 237, 240]. In the United States, a multi-exon deletion spanning exon 23 though 29 is most frequent, accounting for 28% of detected mutants [224].

Reported mutation detection rates vary from 55 to 80%. Lack of one or both mutations can be due to middle-sized insertions or deletions – often missed with direct sequencing –, mutations in gene regulatory sequences or molecular analysis of patients with a PXE phenocopy. The argument of locus heterogeneity of PXE, although unlikely, cannot be ruled based on these detection rates.

To date, no correlation has been established between the phenotype and the nature or position of the mutations. There is no significant association of mutations leading to a premature stopcodon with a more severe phenotype. Several studies are however hampered by relatively small patient groups and/or ill-defined phenotypes. The high intrafamilial variability is suggestive of other factors, both environmental and genetic modifiers, to influence the PXE phenotype.

Recently, polymorphisms in three genes – *SPP1*, *XYLT-1* and *XYLT-2* – have been suggested as modifiers of the PXE phenotype [241]. The *SPP1* gene encodes osteopontin, known to be involved in mineralization. *XYLT-1* and *XYLT-2* encode the xylosyltransferase 1 and 2 enzyme respectively. Both Golgi-resident enzymes catalyze the transfer of xylose from UDP-xylose to serine residues in the proteoglycan core protein and have been identified as risk factors for a.o. diabetic nephropathy and osteoarthritis [242].

1.3.3.3 Other phenotypes associated with *ABCC6*

Only a few authors have attempted a candidate gene approach using *ABCC6* for other – often more common – disorders than PXE. Most studies – including cohorts of cerebral and abdominal aneurysm patients or cervical artery dissections - turned out negative, although the patient group investigated was usually quite small [239, 240].

Naouri et al. looked at *ABCC6* mutations in patients with elastofibroma (EF), a subcutaneous fibroelastic pseudotumour, usually presenting in adulthood at the lower end of the subcapsular space [243]. As in PXE, dystrophic elastic fibres can be observed albeit without mineralization or elastorrhexis. PXE and EF have also been reported once in the same patient [78]. No *ABCC6* mutations were however found in the 3 patients examined.

1.3.4 Pathomechanisms of PXE

Although the pathophysiology of PXE remains largely unknown, two major hypothesis, the "**cell hypothesis**" and the "**metabolic hypothesis**" have been proposed to shed light onto the processes leading to ectopic elastic fibre mineralization.

1.3.4.1 The PXE cell hypothesis

In the "PXE cell hypothesis", it is postulated that absent expression of *ABCC6* in fibroblasts or smooth muscle cells, may result in local mineralization as a result of cellular perturbation. The hypothesis is based on experiments on fibroblast cultures established from skin lesions of PXE patients. These cells have been suggested to display abnormalities in cell-cell and cell-matrix adhesion properties, in the proliferative capacity of the cells harbouring the mutations and in the rate of synthesis of connective tissue compounds, such as elastin, collagen and proteoglycans [244]. The cells have been shown to suffer from mild chronic oxidative stress and to demonstrate increased degradative potential as reflected by elevated MMP-2 expression [245, 246]. Question remains whether the observed mild oxidative stress is an important primary pathogenetic mechanism or merely a secondary end-stage result of ECM degradation in PXE.

1.3.4.2 The PXE metabolic hypothesis

The metabolic hypothesis is based on the clinical observation that PXE is a slowly progressing disorder, caused by a transporter, predominantly expressed in the liver and kidney, which apparently has transport capacities from intracellular towards the blood stream. Hence, it has been postulated that in the absence of functional ABCC6, the levels of certain metabolic compounds in the circulation are altered. The perturbed levels of such compounds may affect the connective tissue directly, may have an indirect effect through pathways involved in ECM homeostasis or may alter the biosynthetic capacity of resident cells, thus leading to pathologic mineralization and degradation. The findings in *Abcc6* knockout mouse models, described below, support the hypothesis that PXE is a metabolic disease with secondary connective tissue manifestations [247].

Le Saux et al. addressed this hypothesis by incubating cultured fibroblasts from the skin of patients and age-matched, unaffected individuals in medium supplemented with normal human serum or serum from patients with PXE [248]. Results indicated that in the presence of normal human serum, PXE fibroblasts displayed increased synthesis of elastic fibres in comparison with normal fibroblasts, but the fibres were structurally normal. When maintained in the presence of serum from PXE patients, both PXE and normal fibroblasts deposited abnormal aggregates of elastic fibres. It can be concluded that certain metabolites in the PXE serum interfered with normal elastic fibre assembly *in vitro* [248]. Although no data are available of the effects of sera on the mineralization process, these experiments also support the metabolic hypothesis.

1.3.5 Diagnosis

The consensus for the diagnosis of PXE is based on a **clinical and histological evaluation**. Minimum criteria were said to be the presence of middermal elastorrhexis, associated with angioid streaks. In the advent of molecular testing, this consensus – although generally true, particularly in older patients – has been questioned by occasional reports of patients with molecularly confirmed PXE who were missing either skin or ocular involvement [249]. In addition, in children with PXE the ocular phenotype is often non-existing at the time of onset of the skin features. In this respect, molecular analysis of the *ABCC6* gene has been suggested as an alternative diagnostic tool in patients with features of PXE. The cost and labour intensiveness of these analyses being one of the major concerns, several authors have suggested a two- or multi-step approach for *ABCC6* analysis to become more efficient [219, 234]. So far however, such strategies have been of little help in the screening of *ABCC6* outside the specific study-populations of these reports. Hence, no guidelines are available, neither on the position of molecular analysis in the diagnostic work-up of a novel PXE patient nor on its relation towards a skin biopsy.

1.3.6 Differential diagnosis

The differential diagnosis of classic PXE is usually not that difficult, although several other disorders may present with some symptoms considered typical for PXE. Although the skin lesions are characteristic in their appearance and location and can be confirmed through skin biopsy, table 4 lists the most important diseases, both hereditary and acquired, in which the skin features are highly similar to PXE. Interestingly, treatment with D-Penicillamin (DPA) for e.g. Wilson's disease causes a pseudo-PXE, possibly through interference with elastin cross-linking (by inhibiting lysyl oxidase) or by formation of complexes with the cross-linked precursors, impairing a normal maturation of elastic fibres [250]. Skin biopsy however will not show the characteristic elastorrhexis.

Conversely, several disorders such as localized acquired cutaneous PXE (LAC-PXE or periumbilical perforating PXE) or Saltpeter contact may show a histological PXE-like appearance but have only limited clinical resemblance [251, 252]. LAC-PXE is an acquired disorder, most frequently found in obese, multiparous middle-aged women. The main clinical feature is a yellowish, reticulated or cobblestone plaque with papules in the periumbilical region. Patients should be reassured that, although no treatment is available, the disorder is benign and limited to the skin [253-257].

Disorder	Main clinical and/or histological difference(s)	Ref.
White papulosis of the neck &	Flesh-colored papules that occur in the neck but also	[255, 256]
PXE-like papillary dermal elastolysis	in nonflexural sites	[257-259]
Hyperphosphatemic tumoral calcinosis (MIM#211900)	Identical skin features, however without elastorrhexis	[258]
Familial hypoalphalipoproteinemia (MIM#107680)	Infiltrative xanthomas confined to neck and elbows	[259]
Buschke-Ollendorf syndrome (MIM#166700)	Identical skin features, often on trunk, buttocks & feet	[260]
D-Penicillamin treatment	Isolated elastic fibre degradation without calcification	[250]
Elastosis perforans serpiginosa	Clumped elastic fibres extruded through epidermis	[83]
Perforating peri-umbilical PXE	Identical skin features, isolated around umbilicus	[253, 254, 261-265]

Table 4
Dermatological differential diagnosis for PXE

Of the ophthalmological features, AS are not pathognomonic for PXE, with over 20 other disorders in which they have been observed. The most important ones are listed in table 5.

Disorders featuring angioid streaks
Sickle cell anaemia
Beta-thalassaemia
Paget's disease of the bone
Pituitary disorders (acromegaly)
Familial hyperphosphatemia

Table 5
Disorders associated with angioid streaks

A puzzling finding is that patients with inherited **haemoglobinopathies**, most often beta-thalassaemia but also sickle cell disease, may have elastic tissue changes and clinical features closely resembling – if not identical to – PXE. The skin, eye and cardiovascular symptoms are indeed indistinguishable from PXE – except for their age of onset which is usually later in life – despite that none of these patients carry *ABCC6* mutations [266-275]. These clinical findings are not anecdotal in haemoglobinopathy patients: out of a cohort of 100 patients with major or intermediate beta-thalassaemia, 26 patients had angioid streaks and/or skin lesions [272]. While one hypothesis might be that this is related to the pathophysiology of the primary haemoglobinopathy (and as such "acquired"), the identical histological findings in these patients suggest that either a pathway is involved which is independent of *ABCC6* or that the largely unknown *ABCC6* pathway is affected more downstream.

Besides the haemoglobinopathy phenocopy, there have recently been two reports of **unclassified autosomal dominant conditions** in large families, resembling PXE, in which involvement of the *ABCC6* gene was excluded by linkage analysis. In one three generation family, affected individuals had mottled fundi, angioid streaks and drusen in various combinations, but no skin involvement suggestive for PXE [276]. Robert et al. described a four generation pedigree in which patients had typical skin involvement with papules and skin laxity as well as angioid streaks in fundo. Interestingly, only one patient showed mid-dermal elastorrhexis on skin biopsy, while the other affected individuals had a normal mid-dermis [277]. Both disorders, of which the molecular background remains currently unknown, might be examples of the clinical spectrum which we call PXE.

1.3.7 Animal models

An animal model for PXE has been long considered to be useful in pursuing a better understanding of the pathophysiology, not only because of the unclear etiological role of ABCC6 but also because of the difficulty of tissue availability for functional analysis. Indeed, besides skin, the other frequently affected organs (eye and cardiovascular system) are more difficult to obtain because of debilitating biopsy effects (eye) or lack of need for surgery in PXE (CVD). The same is true for the organs – liver and kidney – which most significantly express ABCC6. As all of the human ABC genes have mouse homologues, besides *ABCC11* (which may have risen as a recent duplication of *ABCC12*), the mouse presents an excellent animal model system to study ABC genes.

Towards the development of an animal model for PXE, Uitto et al. generated a **targeted mutant mouse** in which both alleles of the *Abcc6* gene were inactivated by targeted ablation [278]. The histological phenotype of these mice revealed mineralization in various connective tissues, although with distinct differences compared to humans. The accumulation of calcium in the middermis is very focal compared to humans. Mineralization was particularly dense at the periphery of the fibres, while in PXE patients the calcifications are located in the elastic fibre core. Remarkably, severe calcification of the collagen fibres was also observed. Abnormal collagen fibrillogenesis, leading to cauliflowers has been observed in PXE tissues; however, these fibres do not tend to be mineralized. A striking feature was the mineralization of the connective tissue capsule surrounding the bulb of the vibrissae, which is detectable at 5-6 weeks and progressively increases. Although this has been suggested as an early biomarker of the pathologic mineralization process, its relevance for human PXE remains debatable. Immunohistochemical characterization of the mice demonstrate direct association of fetuin A, MGP, Ank and APase activity with the mineralization process of the calcified vibrissae [278].

A second mouse model, created by **targeted disruption of *Abcc6***, results in extensive mineralization of soft tissue – including the skin, retina and blood vessels, in homozygous mice, whereas the heterozygous animals are indistinguishable from wild type mice [279].

From a clinical perspective, no convincing skin lesions could be seen besides very small and largely dispersed papules. Similarly, no data on features of the retinopathy in *Abcc6 -/-* mice are available, despite calcification in BrM. These findings suggest that, although present mouse models may serve as a model for ectopic mineralization, one must remain critical and careful in extrapolating findings to human PXE.

Recently, *Abcc6* has been identified as the major causal gene for dystrophic cardiac calcification (DCC) in mice [280-282]. Using a mouse model, the *Dyscalc1* locus was fine-mapped and by integrating genetic segregation and expression array analysis, a single candidate gene, *Abcc6,* was identified. It was demonstrated that in the DCC model, the expression of *Abcc6* is highly correlated with the local mineralization-regulatory system and the BMP2-Wnt signalling known to be involved in the systemic regulation of calcification [280]. Although myocardial calcification has not yet been reported as a phenotypic feature in human PXE, nor in the *Abcc6-/-* models, these findings might suggest potential pathways for the action of *ABCC6*.

1.3.8 Prognosis and treatment

1.3.8.1 Prognosis

Data on the natural course of the skin, eye and cardiovascular features are limited. Hence, any prediction on the prognosis of PXE is based on a limited number of observations reported in literature. The absence of genotype-phenotype correlations makes it currently impossible to individualise prognostic counselling, while recently identified potential modifiers await further study to evaluate reproducibility before they can be used in the clinic. It must be noted however that the prognosis of PXE in terms of life expectancy and life quality strongly depends on the age of diagnosis. When diagnosed at a young age, preventive measures and current therapeutic options may assure that the life span of a PXE patient is equal to the general population and that quality of life is maintained at a high level.

1.3.8.2 Treatment

At present, there is no treatment to “cure” PXE. Management focuses on prevention of complications and – if necessary – early intervention when they do occur. Unfortunately, no standard guidelines are available for the care of PXE families.

a/ prevention

Prevention of subretinal haemorrhages comprises avoiding ball and contact sports, to reduce the risk of facial trauma. Similarly, isometric activities (weight lifting, …) are contraindicated. All patients are advised to perform a regular self-examination to detect metamorphopsia using an Amsler grid (Figure 16). If the straight lines of this grid are seen as undulating lines, patients are asked to come into the hospital immediately for treatment. Practical alternatives to the Amsler grid are the tiles in kitchen or bathroom and the subtitles on television.

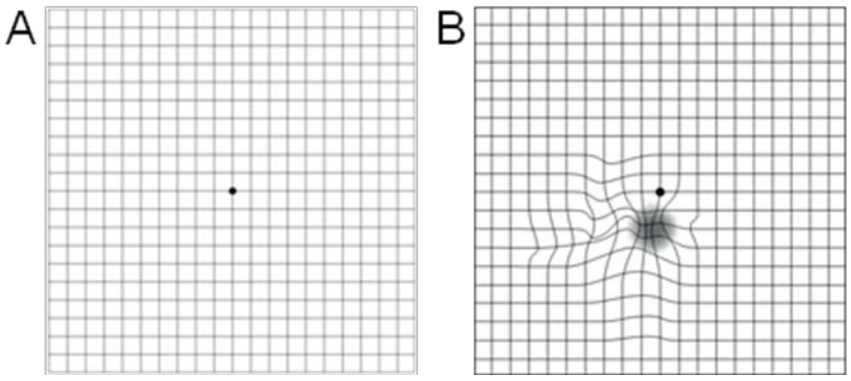

Figure 16
Amsler grid as observed by a normal individual (A) and as observed by a PXE patient with metamorphopsias (B)

Cardiovascular prevention focuses on classic risk factors such as hypertension, hypercholesterolaemia, diabetes, obesity and smoking. Aspirin and non-steroidal anti-

inflammatory drugs should be avoided because of the risk for mucosal bleeding. Particularly in patients who suffered an ischemic stroke, this can cause a significant therapeutic dilemma, for which no optimal solution presently exists.

A single study has addressed the role of calcium restriction in the diet and found it to have a beneficiary influence on the evolution of PXE by reducing the extent of mineralization. However, the poor methodology of this study resulted in many authors refuting these findings. As such, there is presently no evidence that dietary measurements have any efficacy in PXE prevention.

b/ treatment

Surgical reduction of excessive and redundant skin for cosmetic improvement has been applied in some patients. The long term outcome is however highly variable and cases are mostly anecdotal. Several difficulties, such as delayed wound healing and scarring, should be taken into account, as well as the re-occurrence of skin folds [283]. The efficacy of collagen or autologous fat injections in the mental creases has not been established [284]. Preliminary studies with aluminium hydroxide, an oral phosphate binder, have suggested a significant clinical improvement of skin lesions and absence of ophthalmological deterioration [285]. The study cohort was however quite small (6 patients) and the follow-up period limited (1 year). Hence, these observations should be confirmed in a larger patient group before clinical use.

Several approaches have been used in the past to stop vessel proliferation. Laser therapy was effective in some patients with submacular neovessels, but causes retinal burns and subsequent scotomata [286]. Also, a recurrence rate of 50% has been noted [286]. Surgical procedures, such as macular translocation for subfoveal choroidal neovascularisation, have occasionally been undertaken [287]. Because of the resemblance of the PXE retinopathy with age-related macular degeneration, photodynamic therapy and – more recently – anti-angiogenic drug therapy have been attempted. The former consist of a peripheral injection of *verteporfine*, a drug known to undergo a conformational change when targeted with deep red light, forming a clot. When targeting specifically the neovessels, clotting will induce anoxia and shrinking of the vessel thus preventing subretinal haemorrhage. Results of this approach are variable between patients – from significant improvement of visual acuity to stabilisation of decreased vision – and patients must avoid direct exposure to sunlight [288-290]. Red light is present in sunlight and, as the verteporfin – with a half-life of 72 hours – is present throughout the body after infusion, could induce vessel clotting throughout the body. The latter consist of intra-ocular injection of anti-VEGF antibodies (Bevacizumab or Avastin®, Pegaptanib Sodium or Macugen®, Ranibizumab or Lucentis®), which block the trigger inducing the formation of neovessels, leading to shrinkage. Current results show good outcome – preservation of normal visual acuity – in patients with metamorphopsia but without haemorrhage [291, 292]. When haemorrhage has occurred, an improvement of vision can be envisaged. Recent reports of possible systemic side-effects emphasized the necessity of performing this treatment under well-controlled circumstances and the need for larger clinical trials.

Despite the occurrence of gastro-intestinal bleeding, it is noteworthy that no vascular brittleness exists in PXE. As such, cardiovascular complications can be treated by standard surgical or interventional radiological neovascularisation procedures if indicated. For coronary surgery, controversy exists on whether the left internal mammary artery, which may be involved in PXE is suitable for bypass grafting [293-295].

Chapter 2
Patients, materials and methods

"Chaque jour, à dix heures, mon médécin vient me prendre ma température et m'en donne une nouvelle."

Erik Satie

The Center for Medical Genetics of the Ghent University has developed a specific expertise in the study of hereditary CTD, which is unique in Belgium and Europe. At the clinical level, a multidisciplinary setting was created for patients with hereditary CTD. In addition, a laboratory for biochemical and molecular research into the genetic defects in connective tissue proteins, such as the collagens and elastin is present. From its position as a health care service, a unique opportunity has been created to expand the research in this domain and to implement new developments in the clinic. By elucidating the genetic basis of this complex group of disorders, it is attempted to offer better diagnostics, prevention and new perspectives for treatment.

2.1 Patient population

PXE patients and family members were gathered from our own PXE clinic, from several European countries and from the registry of PXE International, a large patient support group offering a DNA- and tissue databank of over 3000 patients. Clinical data were gathered either through detailed clinical checklists – filled out by the referring physician – or by personal physical examination.

In our PXE clinic, all patients are submitted to a clinical protocol consisting of:

1/ a **skin biopsy**, which is taken either in a skin lesion or at the lateral side of the neck when no lesion is clinically apparent. Histological confirmation of PXE is obtained with haematoxylin & eosin, Van Giesson and von Kossa stains.
In some of these patients, part of the biopsy was processed for electron microscopy evaluation and/or immunohistochemical experiments.

2/ a thorough **macroscopic skin evaluation**. To establish potential genotype-phenotype correlations, dermatological manifestations are scored as follows: S0: No typical skin lesions; S1: Yellow papules; S2: Plaques of coalesced papules; S3: Laxity and redundancy of skin.

3/ measurement of **best-corrected visual acuity**, combined with dilated fundoscopy and fundus photography to evaluate the PXE retinopathy. The ocular manifestations are scored as following: E0: No fundus abnormalities; E1: Peau d'orange; E2: Angioid streaks; E3: Subretinal neovascularisation with retinal haemorrhages; E4: Macular degeneration with scarring.

4/ with respect to the **cardiovascular history**, information on intermittent claudication, vascular occlusion requiring surgery, angor pectoris or myocardial infarction is obtained. Physical exam specifically screens for presence of weak or absent pulses and hypertension. Echocardiography looks for the presence of mitral valve prolapse and mild or severe valvular regurgitation. Finally, Doppler examinations of the carotid and femoral arteries are performed.

5/ an **ultrasonographical examination**, detailed below, to evaluate calcium precipitation in the abdominal organs (in particular liver, spleen and kidneys) and the testicles.

6/ a **blood sample** of EDTA (for DNA extraction), serum (for routine liver and kidney tests, ionogram and ELISA tests) and/or citrate (for clotting factor analysis and ELISA tests).

A nearly identical examination protocol, aside from the skin biopsy, is always proposed for family members in whom molecular analysis confirmed carriership of 1 *ABCC6* mutation.

The scores for skin and ocular manifestations were delineated from the Phenodex, a scoring system developed by PXE International (see *publication 2*). Unfortunately, such a scoring method is always artificial, as multiple signs and symptoms within one category – skin, eyes or cardiovascular system – can occur at the same time in one patient. This is particularly true for the cardiovascular complications, which is the reason why I did not use such a scoring system for the cardiovascular history. Due to these shortcomings, the Phenodex has limited use as a descriptive tool for the PXE phenotype. However, it does provide sufficient simplification to perform statistical analysis for phenotype-genotype correlations.

2.2 Methods

2.2.1 Clinical methods and techniques

Besides the clinical examination, as described above, the clinical techniques most frequently used in this thesis are ultrasonography, visual electrophysiological examinations and auto-fluorescence imaging of the fundus.

2.2.1.1 Ultrasonography

Ultrasound is the form of mechanical vibrations in which the vibration has a frequency in excess of that to which the human ear is sensitive (20-200.000 MHz). It is produced by a transducer made from a piezoelectric material, capable of both transmitting and receiving waves. The generated ultrasonic waves are reflected from naturally occurring bounderies between different tissues within the body, a fraction of the energy being reflected if there is impedance at such boundary. Energy that is not reflected travels onwards, with the depth to which it can penetrate being limited by the attenuation of the wave.

In an **A-scan** (or amplitude mode), the transducer is pulsed and the echoes received from each tissue interface are displayed as spikes on an oscilloscope screen. The distances between spikes are a measure of the separation debt of the reflecting tissues whereas spike heights indicate echo amplitudes. In a **TM-scan** (time and motion), the spikes are registered as moving dots in a line on the screen. Motions can be detected by software and correlated with the echoes, locating their origin and assembling the data to form a two-dimensional gray-scale image or **B-scan**. **Doppler** imaging, based on the Doppler principle by which a perceived shift in frequency occurs when a sound-producing object passes an observer, is used to measure the motion of blood or other fluids through a vessel.

2.2.1.2 Visual electrophysiology

Visual electrophysiology aims to objectively test the function of several components of the human visual system. Several tests are available, of which only **full-field flash and pattern electroretinography (ERG)** will be discussed briefly. All tests were performed in accordance with standards and/or guidelines suggested by the International Society for Clinical Electrophysiology of Vision (ISCEV) [296, 297].

a/ neuroretinal anatomy

The **neurosensory retina**, evaluated by the techniques below, consists of three types of neurons: the photoreceptors, bipolar cells and ganglion cells (Figure 17).

The **photoreceptors** are divided in two distinct classes, the rods and the cones. So named because of their different morphology, these cells, responsible for the translation of light

energy into an electrical signal, are also very different in the way they function. The rods are very sensitive to light and therefore responsible for night vision (scotopic). Cones require higher amounts of ambient light (photopic) to be activated and mediate high spatial resolution and colour vision.

Rods and cones synapse with the second order neuron in the retina, the **bipolar cells**, which form a relay with the retinal ganglion cells (or third order neurons). Via their axons, information is conveyed through the optic nerve to the occipital visual cortex.

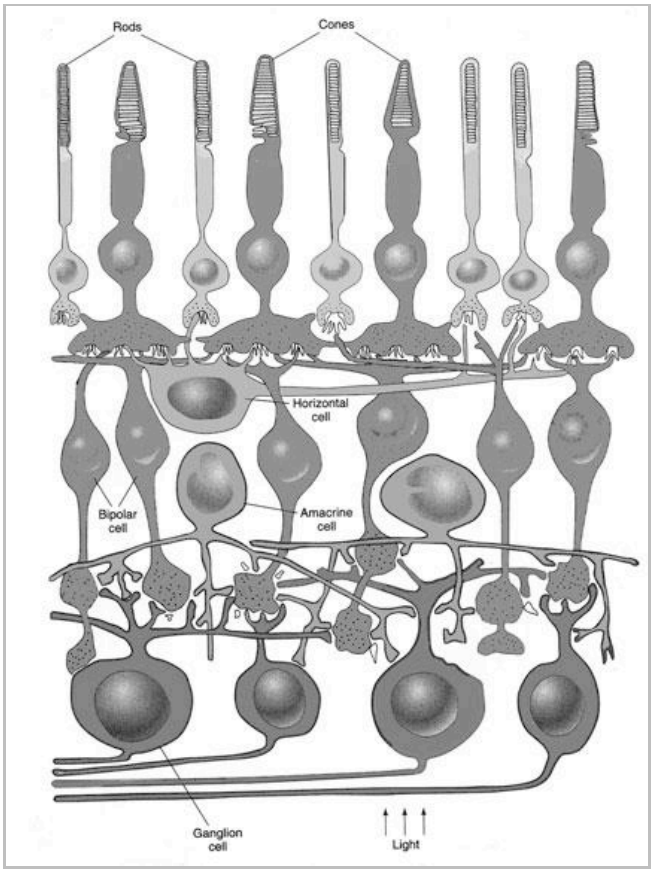

Figure 17
Schematic representation of the neuroretinal anatomy

b/ full-field flash ERG

The full-field flash ERG records a mass electrical response generated by both the neural and nonneural retinal cells of the **whole retina**, upon stimulation of the eye with a flash of light. It therefore provides an objective measure of retinal function without the need for too much cooperation by the patient.

After the patient is dark-adapted for a minimum of 20 minutes, a whole field stimulator is applied to stimulate the whole retina through dilated pupils. As such, 5 different standard responses are generated:

1/ ERG to weak flash from the dark-adapted eye, elicited by the **rod photoreceptors**;
2/ ERG to a strong flash from the dark-adapted eye, arising from the **rod and cone photoreceptors**;
3/ Oscillatory potentials, arising from the **inner retina.**

After ten minutes of light adaptation, the remaining two responses are generated:

4/ ERG to a strong flash in the light-adapted eye, arising from the **cone photoreceptors**;
5/ ERG to a rapidly repeating stimulus (30Hz), arising from the **cones.**

Gold foil electrodes, recording electrical activity of the retina, are placed onto the cornea. Final traces are usually averaged to minimize interference by artefacts.

The classic ERG trace has two distinct components, generated by different cell layers of the retina (Figure 18). An initial negative deflection is called the **a-wave**, which is followed by a positive **b-wave**. The a-wave is generated at least partially by the photoreceptors, the b-wave reflects activity of bipolar and Müller cells (supportive cells of the retina). Several characteristics which can be evaluated include the amplitude and latency of the a-wave and the amplitude and implicit time (from stimulus onset to peak) of the b-wave. Abnormalities in any of the former allow better characterisation of a clinical phenotype. The standard ERG protocol thus allows us to make a distinction between the response of the rods and that of the cones and between involvement of the outer retina (a-wave) versus that of the inner retina (b-wave).

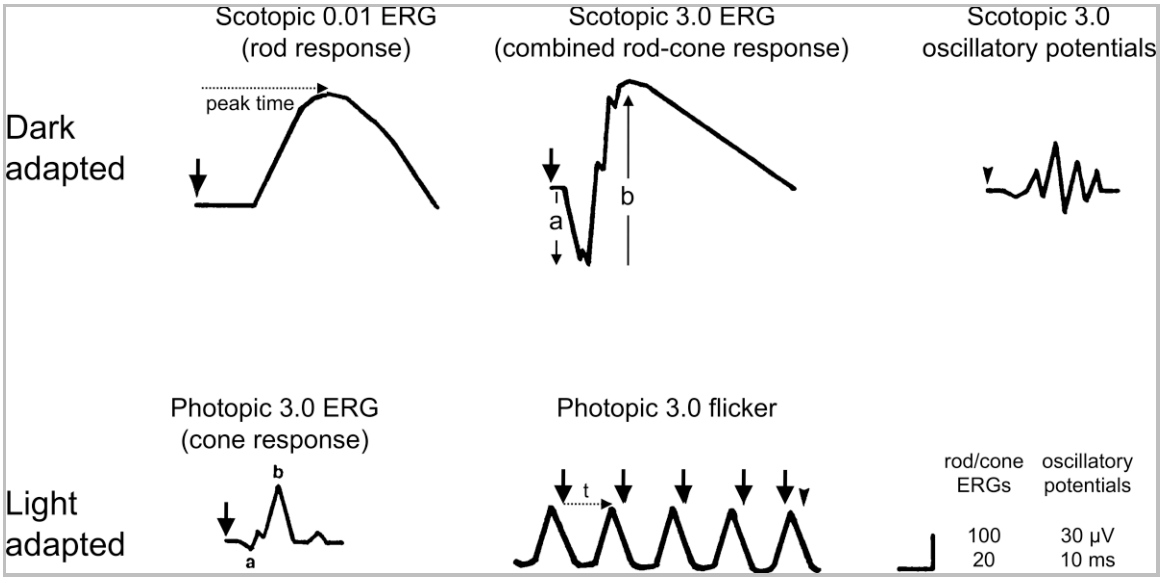

Figure 18

Normal full-field flash electroretinographical trace. The waveforms are exemplary only, and are not intended to indicate minimum, maximum or even average values. Large arrowheads indicate the stimulus flash. Dotted arrows exemplify how to measure time-to-peak (*t,* implicit time), *a*-wave amplitude and *b*-wave amplitude.
Adopted from Marmor et al. [297]

c/ pattern ERG (PERG)

PERG is a retinal response elicited from the **macular area of the retina**, traditionally using a transient pattern reversal checkerboard stimulus. It is measured in photopic conditions with optimal refraction and undilated pupils. Electrode positions are similar to those for full-field ERG. A typical PERG response consists of an initial small component at 35 ms of stimulus onset (N_{35}), a positive deflection at 50 ms from stimulus onset (P_{50}) and a subsequent negative component at 95 ms (N_{95}) (Figure 19).

Whereas the full field ERG does not convey any information on the retinal ganglion cell layer, the PERG does so. Therefore, the combined use of full field ERG and pattern ERG explores the response of all retinal cell layers.

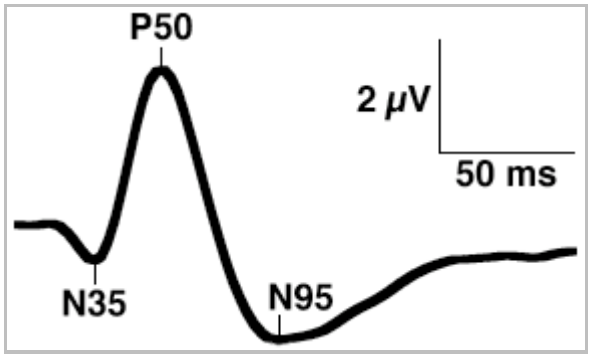

Figure 19
Normal pattern ERG trace
Adopted from Marmor et al. [297]

2.2.1.3 Autofluorescence imaging

Besides white light digital photography, fundus imaging was performed using fundus **autofluorescence** (AF), **red free** and **infrared** monochromatic images. These novel techniques offer new ways of identifying various manifestations of retinal disease. Using a confocal scanning laser ophthalmoscope (cSLO), AF shows the distribution of **lipofuscin** in the RPE *in vivo*. This pigment accumulates in the RPE with aging as a result of incomplete photoreceptor degradation. Because of its suggested role in senescence, the detection of lipofuscin has been considered a noninvasive marker for monitoring the status of the RPE [298].

After pupillary dilatation with tropicamide 1%, autofluorescent images were recorded using argon blue laser light for excitation and a barrier filter with a cut off at 500 nm to record fundus autofluorescence and red free imaging. For near infrared imaging, wavelengths are 787 nm for excitation and a barrier filter allowing light passage above 810 nm. Full-emission spectra are recorded via a polarization filter to obtain red-free and infrared images. Before acquisition of the autofluorescence sequence, illumination and focus level are adjusted to individual requirements at the infrared mode of the device, in order to generate high quality images. Infrared, autofluorescence and red free images, in series of approximately 50 single pictures per eye, are generated using a 30-degree field-overview mode. The images encompassed the entire macular area, retinal midperiphery and periphery. After automated alignment and averaging to improve signal to noise ratio by image analysis software (Heidelberg Eye Explorer, Heidelberg

engineering, Dossenheim, Germany) mean images are used for further analysis. Finally, HRA 2® software can be applied on selected individual and mean images of excellent quality to generate a seamless montage of the entire fundus in AF, RF and IR mode.

2.2.2 Molecular methods and techniques

2.2.2.1 Amplification of DNA sequences

In order to analyse a DNA sequence of interest, molecular techniques require an amount of DNA that is usually not readily available from patients. To make a high number of copies of the sequence of interest in the patient's DNA, the **polymerase chain reaction (PCR)** was used. Routine PCR can amplify fragments sized up to 5 kb; because of the presence of *ABCC6* pseudogenes, larger fragments needed to be amplified to exclude these pseudogenes. To this end a long-range PCR method was used. A PCR or long-range PCR reaction consists of 30 to 40 fully automated cycles of three basic steps (Figure 20):

1/ heating to melting temperature (95°) to obtain single-strand DNA;

2/ annealing of forward and reverse sequence-specific primers;

3/ primer extension, using nucleotides and based on the sequence of the DNA template obtained after step 1.

A final elongation step after the last cycle at 72° ensures proper completion of the structure of all molecules.

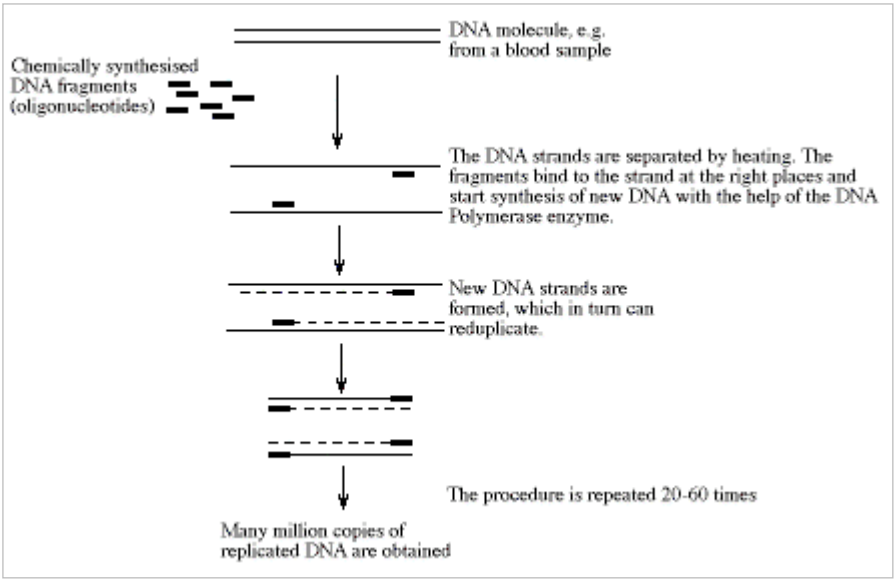

Figure 20
Schematic representation of the polymerase chain reaction

2.2.2.2 Mutation detection

To determine whether a mutation is present in a fragment of DNA, its sequence can be determined using **sequencing**. The sequencing technique is based on enzymatic single strand DNA synthesis with base-specific dideoxynucleotides, serving as chain terminators (Figure 21).

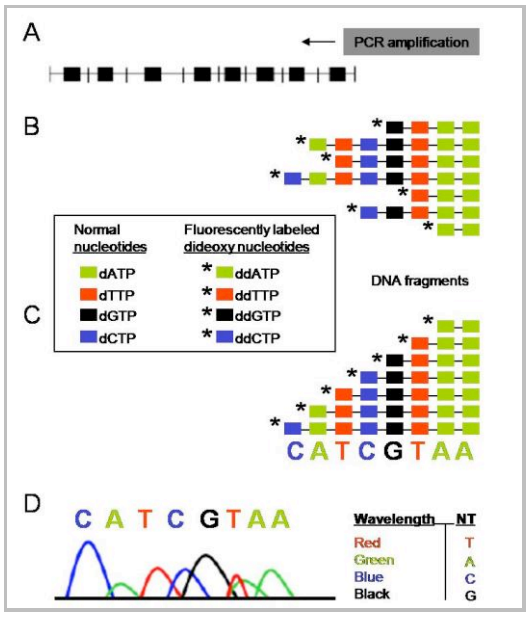

Figure 21

The principle of direct DNA sequencing. Single strand DNA is obtained and amplified using a PCR reaction (panel A). Panel B and C display the mixture of ss-DNA molecules, varying in length by one base and terminated with a fluorescent dideoxynucleotide. Panel D shows the output, an electropherogram, after passing through a scanning system.

A single-stranded fragment of patient DNA acts as a template to form a mixture of single-stranded DNA molecules, all varying in length by one base. Each molecule is terminated with one dideoxynucleotide with a base-specific fluorescent signal. The latter is picked up by a laser-based scanning system and is translated into a base sequence for the particular fragment. The graphical representation, obtained by the sequencing software, is called an **electropherogram** and depicts the two alleles of the gene of interest. As both are identical in physiological circumstances, the alleles are superimposed and only one peak is observed for every nucleotide. In case of a heterozygous mutation, a double peak can be observed while homozygous mutations give rise to a single peak of the incorrect nucleotide.

High throughput facilities have made direct sequencing more efficient and economic than before. At the beginning of this project, the technique was however still laborious and a less arduous screening procedure, **denaturing high-performance liquid chromatography (dHPLC)** was applied (Figure 22). During the last two years of this project, the technique has been abandoned in the advent of high throughput analysis techniques.

First, the DNA fragments under study are denatured and then reannealed with single-stranded wild-type DNA fragments to form either homo- (when no sequence change is present) or heteroduplexes (in the presence of a sequence change). dHPLC is based on the differential

retention of these homo- and heteroduplexes in the liquid chromatography column. Variations in temperature and chemical composition of the elution buffer were standardised for each exon of a specific gene. The generated data are interpreted in a graphical way; samples displaying an altered curve compared to the wild type are further investigated through sequencing.

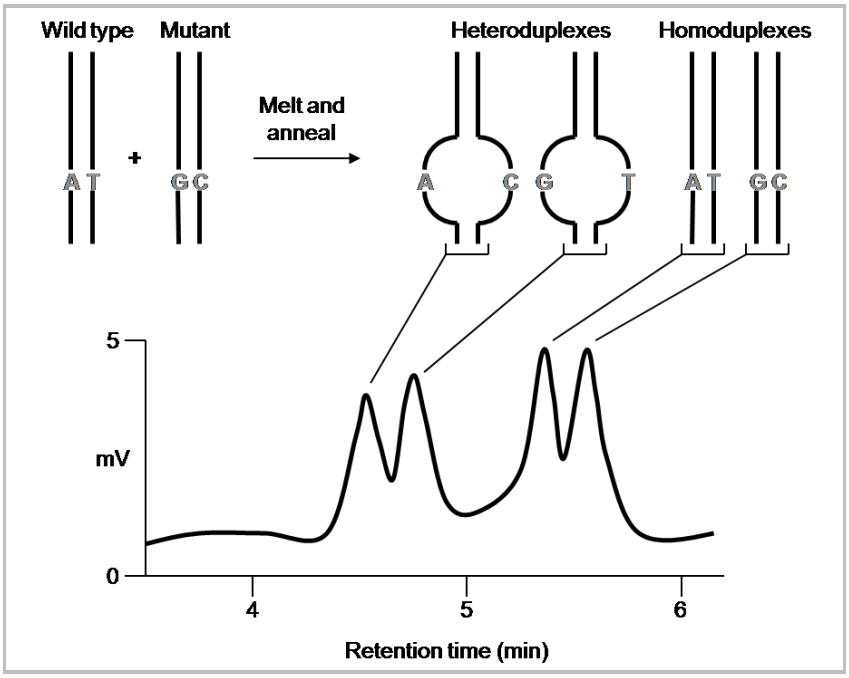

Figure 22
The dHPLC principle, based on the detection of heteroduplexes

One of the major challenges in mutation analysis is the substantiation of causality of a given base pair change. In the event of deletions, insertions, frameshifts or when a premature termination codon is introduced – when a nonsense mutation occurs – causality is easily assumed. For missense mutations, causality is often not straight forward and a series of criteria can be used to predict whether a change may be pathologic. These include conservation of the affected amino acid between different species (or orthologs) and/or between different members of the same protein family (or paralogs). Also a change in biochemical characteristics of the mutated amino acid can suggest causality. Next, several *in silico* analysis techniques are available to predict the pathologic nature of a sequence variation. Finally, if a base pair change is not found in 200 control alleles, this increases chances of it being a causal mutation.

2.2.3 Biochemical methods and techniques

For the detection of various antigens in tissues, serum or plasma, we applied **immunohistochemical** and **Enzyme-Linked Immunosorbent Assays (ELISA)** using antibodies provided by VitaK®, a spin-off of the University of Maastricht (The Netherlands). Both techniques are basically analogous, if not that they use test samples of different origin. The technical details of these experiments are explained in chapter 3; here, only the general principles of these techniques will be discussed.

a/ general principles and terminology

The techniques described below are based on one of the basic concepts of immunology, namely the specific immune response or the production of an antibody to a particular antigen.

An **antibody** is a plasma molecule that binds specifically to particular proteins; in innate immunity these are able to bind and neutralize pathogens while in the immunological techniques described here they are used to capture, measure and/or visualize a protein of interest. For this reason, antibodies can be manufactured *in vitro.* **Monoclonal** antibodies are produced by a single clone of cells, while **polyclonal** antibodies result from several clones, often from more than one species.

An **antigen** is a molecule that reacts with an antibody and named for their ability to *gen*erate *anti*bodies. However, not all antigens – such as those described in this thesis – necessarily elicit such antibody production.

b/ immunohistochemistry

Immunohistochemistry is a method to detect a protein on tissue sections in which the antibody is chemically coupled to an enzyme that converts a colourless substrate into a colored reaction product whose deposition can be directly observed under a light microscope.

c/ enzyme-linked immunosorbent assays (ELISA)

For an antigen (e.g. a protein of interest) to be detected, a purified antibody specific for this antigen is linked to an enzyme. The samples (plasma or serum) to be tested are coated onto the surface of plastic wells to which they bind non-specifically. The labelled antibody is then added to these wells under conditions where non-specific binding is prevented, so that only binding to the targeted antigen causes the labelled antibody to be retained on the surface. Unbound labelled antibody is removed from all wells by washing, and bound antibody is detected by an enzyme-dependent colour-change reaction, using spectrometers.

This basic serological assay has been modified to measure directly the amount of antigen or antibody in a sample of unknown composition, by using competitive inhibition or sandwich assay (Figure 23).

a/ with a **competitive ELISA** the presence and amount of a particular antigen in an unknown sample is determined by its ability to compete with a labelled reference antigen for binding to an antibody attached to a plastic well. By adding varying amounts of a known, unlabeled standard preparation, a standard curve is constructed and the assay can then measure the amount of antigen in unknown samples by comparison to the standard. This assay can also be used for measuring antibody in a sample of unknown

composition by attaching the appropriate antigen to the plate and measuring the ability of the test sample to inhibit the binding of a labelled specific antibody.

b/ in a **sandwich or capture ELISA**, the antigen of interest in characterized by its ability to bridge between two monoclonal antibodies reacting with different epitopes (or site on an antigen recognized by an antibody) on the antigen. The first, unlabeled antibody is attached to the plastic well and then the test sample is added. After washing, bound antigen is detected by binding a second, labelled antibody directed at a different epitope. This assay is highly specific because antigens that cross-react with one antibody are very unlikely to cross-react with the other.

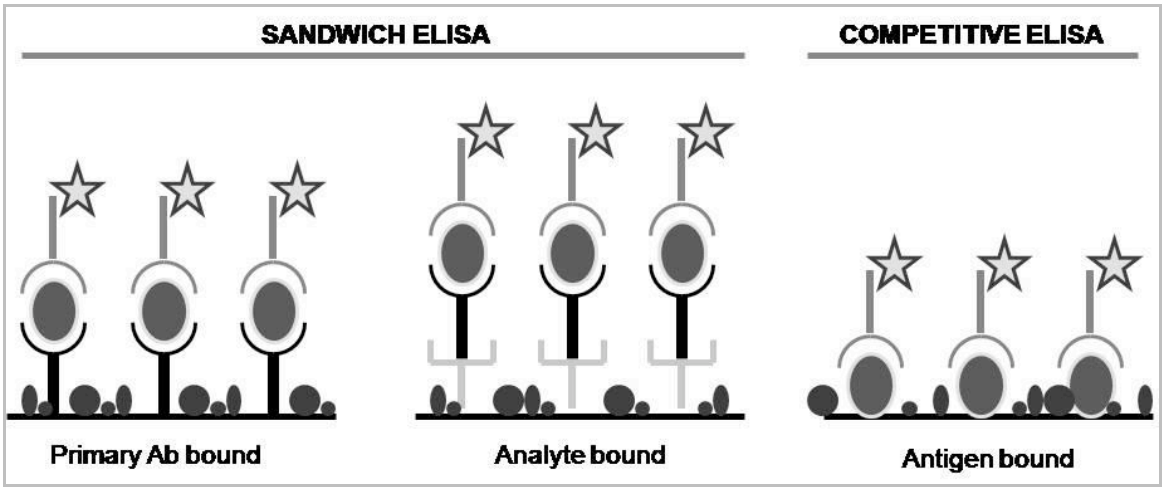

Figure 23
Sandwich ELISA versus competitive ELISA. The analyte is represented by the oval, while the antibodies marked by a star have fluorescent characteristics

Chapter 3
Results

"Une accumulation de faits n'est pas plus une science qu'un tas de pierres n'est une maison"

Henri Poincarré (La science et l'hypothèse)

As the attentive reader will perceive, papers are not always given here in their original order of publication. Rather than merely presenting a cumulative data set, it has been attempted to order the papers into three major topics – molecular studies, phenotypical papers and studies on the PXE-like disorder and its implications for the pathogenesis of PXE. In doing so, this chapter already attempts to integrate all data accumulated in the past four years, which will be further clarified in the discussion chapter.

3.1 *ABCC6* mutational spectrum and genotype-phenotype studies

Publication 1

Novel clinico-molecular insights in Pseudoxanthoma Elasticum provide an efficient molecular screening method and a comprehensive diagnostic flowchart.

Olivier M. Vanakker, Bart P. Leroy, Paul Coucke, Petra Van Acker, Dirk Matthys, Bart Loeys, Anne De Paepe.

Human Mutation 2008;29:205

In this paper, a comprehensive clinical and molecular study of 38 Belgian PXE probands and 21 relatives (4 affected and 17 carriers) is described. An extensive clinical evaluation protocol, described in chapter 2, was implemented and molecular analysis of the *ABCC6* gene performed.

The objective of this study was to itemize the clinical characteristics in both patients and carriers in a thorough and systematic way, with serial assessments over time and correlate them to the molecular data observed in this cohort. As such, this study has contributed to:

a/ a more clear delineation of the "**classic clinical features**" of PXE;

b/ the recognition of **stroke** as a major complication in PXE patients;

c/ formulating easy-to-use **diagnostic flow-charts**;

d/ the expansion of the *ABCC6* **mutation spectrum**;

e/ the unequivocal proof of the **autosomal recessive inheritance** of PXE;

f/ a more **efficient screening method** for the *ABCC6* gene;

g/ the awareness of complex **disease modifying mechanisms** being present.

MUTATION IN BRIEF

Novel Clinico-molecular Insights in Pseudoxanthoma Elasticum Provide an Efficient Molecular Screening Method and a Comprehensive Diagnostic Flowchart

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Communicated by Peter Byers

Pseudoxanthoma elasticum (PXE) is a heritable connective tissue disorder characterized by ocular, cutaneous and cardiovascular manifestations. It is caused by mutations in the *ABCC6* gene (chr. 16p13.1), encoding a transmembrane transporter protein, the substrate and biological function of which are currently unknown. A comprehensive clinical and molecular study of 38 Belgian PXE probands and 21 relatives (4 affected and 17 carriers) was performed. An extensive clinical evaluation protocol was implemented with serial fundus, skin and cardiovascular evaluation. We report on 14 novel mutations in the *ABCC6* gene. We observed extensive variability in severity of both cutaneous and ocular lesions. The type of skin lesion however usually remained identical throughout the evolution of the disorder, while ophthalmological progression was mainly due to functional decline. Peripheral artery disease (53%) and stroke (15%) were significantly more prevalent than in the general population (10-30% and 0.3-0.5% respectively). Interestingly, we also observed a relatively high incidence of subclinical peripheral artery disease (41%) in our carrier population. We highlight the significance of peripheral artery disease and stroke in PXE patients as well as the subclinical manifestations in carriers. Through follow-up data we gained insight into the natural history of PXE. We propose a cost- and time-efficient two-step method of *ABCC6* analysis which can be used in different populations. Additionally, we created a diagnostic flowchart and attempted to define the role of molecular analysis of *ABCC6* in the work-up of a PXE patient. © 2007 Wiley-Liss, Inc.

KEY WORDS: pseudoxanthoma elasticum; *ABCC6*; natural history; diagnostic flowchart

INTRODUCTION

Pseudoxanthoma elasticum (PXE; MIM# 264800) is an autosomal recessive systemic disorder characterized by abnormalities of the skin (papular lesions and increased laxity in flexural areas), the ocular system (peau d'orange and/or angioid streaks with subretinal neovascularisation, haemorrhage and loss of central vision) and the

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cardiovascular system (accelerated atherosclerosis) [Neldner, 1988; De Paepe et al., 1991; Hu et al., 2003]. Its prevalence is estimated to be 1:75000 [Hu et al., 2003].

A clinical diagnosis of PXE is traditionally confirmed by demonstrating fragmentation and calcification of elastic fibres in a lesional skin biopsy, using appropriate staining methods. Although the elastolysis is characteristic, additional abnormal morphology or distribution of other extracellular matrix components (collagen, fibrillins, proteoglycans) is also observed [Lebwohl et al., 1993; Baccarani-Contri et al., 1994].

In 2000, *ABCC6* (ATP-binding Cassette C6 - MIM# 603234) was identified as the defective gene in PXE [Bergen et al., 2000; Le Saux et al., 2001]. The gene sequence consists of 31 exons, spanning ~ 73 kb of DNA. *ABCC6* encodes an ATP-dependent transmembrane transporter, the biological substrate of which is as yet unknown. Similarly, the relationship between aberrant ABCC6 activity and extracellular matrix changes in PXE remains to be elucidated. ABCC6 is mostly found at the basolateral cell membrane in liver and kidneys and to a lesser extent in the tissues affected by PXE [Scheffer et al., 2002]. Its cellular location suggests that ABCC6 transports a substrate important for connective tissue homeostasis into the blood, suggesting that PXE may be considered a metabolic disease.

To date, more than 150 mutations in *ABCC6* have been reported [Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000; Struk et al., 2000; Le Saux et al., 2001; Cai et al., 2001; Meloni et al., 2001; Ringpfeil et al., 2001; Le Saux et al., 2002; Hu et al., 2003b; Chassaing et al., 2004; Gheduzzi et al., 2004; Noji et al., 2004; Chassaing et al., 2005; Hendig et al., 2005; Katona et al., 2005; Miksch et al., 2005; Schulz et al. 2005 ; Schulz et al. 2005; Schulz et al. 2006; Kiéc-Wilk et al., 2007]. Most mutations are located at the 3' end of the gene between exons 24 and 30. Two distinct mutations occur more frequently than others. One is the nonsense mutation c.3421C>T (p.R1141X), which, in a homozygous state, leads to complete loss of *ABCC6* function and is more frequent among the European population [Chassaing et al., 2005]. The second is a large deletion spanning exons 23 through 29 and is more prevalent in the United States [Le Saux et al., 2001]. Presently, no clear genotype-phenotype correlations have been established.

Some authors have suggested that peripheral and coronary artery disease can be present in heterozygous carriers [Trip et al., 2002], although no skin and ocular characteristics have been described so far in such individuals [van Soest et al., 1997; Sherer et al., 2001; Trip et al., 2002; Hu et al., 2003; Wegman et al., 2005]. Hence, it remains unclear what the exact nature of (sub)clinical manifestations in carriers, let alone their clinical relevance might be.

In this study we present extensive clinical and molecular data on 42 Belgian PXE patients (38 probands and 4 affected siblings) and 17 carriers.

PATIENTS AND METHODS

Patient group

Clinical evaluation and analysis of the *ABCC6* gene was performed in a cohort of 38 clinically proven PXE probands, 4 affected siblings, as well as 17 carriers. In 37 probands, a clinical diagnosis of PXE patient was based on the presence of both of the following criteria:

1) ophthalmological manifestations including at least retinal peau d'orange lesions and/or angioid streaks;
2) skin involvement: macroscopic skin lesions including yellowish papules and/or plaques of the skin in the neck, and other flexural areas (armpits, elbows, knees) and/or microscopic skin lesions on full thickness skin biopsy independent of whether or not these were accompanied by macroscopically visible lesions.

One additional proband (pat 30-001) was included based on the presence of angioid streaks, peau d'orange and subretinal neovascularisation with macular degeneration. In addition he suffered from cardiovascular disease (recurrent strokes, myocardial infarction). Family members were considered affected when one of the two previous criteria was present.

The patient population consisted of 17 men and 25 women. Ages ranged from 18 to 81 years (average age of 52 years). The carrier cohort consisted of 11 men and 6 women, with ages ranging from 16 to 76 years (average age of 39 years). Informed consent was obtained from all patients and carriers and the study was approved by the Ethical Committee of the Ghent University Hospital.

Clinical evaluation protocol

All patients were examined by at least one of the authors (OMV, BPL, ADP). Serial fundus, skin and cardiovascular evaluation allowed objective evaluation of progression of disease in 34 patients.

Skin biopsies were taken either in lesional skin or at the back of the neck when no lesion was clinically apparent. Histological confirmation of PXE was obtained with haematoxylin & eosin, Van Giesson and von Kossa stains [Neldner, 1988]. In order to establish potential genotype-phenotype correlations, the dermatological manifestations of patients were scored as follows (adapted from [Gheduzzi et al., 2004]). For macroscopical lesions: S0: No typical skin lesions; S1: Yellow papules; S2: Plaques of coalesced papules; S3: Laxity and redundancy of skin.

Best-corrected visual acuity measurements were combined with dilated fundoscopy and fundus photography to evaluate the retinopathy. The ocular manifestations were scored as following: E0: No abnormalities; E1: Peau d'orange; E2: Angioid streaks; E3: Subretinal neovascularisation with retinal haemorrhages; E4: Macular degeneration with scarring.

With respect to the cardiovascular history, information on intermittent claudication (pain or weakness when walking that is relieved with rest) [Aronow, 2005], vascular occlusion requiring surgery, angor pectoris or myocardial infarction was obtained. Physical exam specifically screened for presence of weak or absent pulses and hypertension (defined as diastolic blood pressure $\geq$ 90 mmHg and/or systolic blood pressure $\geq$ 140 mmHg [O'Brien et al., 2005]. Echocardiography looked for the presence of mitral valve prolapse [Hayek et al., 2005], mild valvular regurgitation (grade I/II) or severe valvular regurgitation (grade III/IV) [Singh et al., 1999]. Finally, Doppler examinations of the carotid and femoral arteries were performed.

Levels of serum calcium and phosphorus, kidney and liver tests and serum lipids (cholesterol [LDL – HDL], triglycerides) were also determined.

Seventeen patients (4 male and 13 female) underwent ultrasonography of the abdomen and testicles. Also, a history of gastro-intestinal bleeding was documented.

A similar protocol was used for the clinical evaluation of obligate carriers. In siblings of patients, molecular analysis was performed to identify carriers prior to a clinical re-evaluation.

Molecular analysis

Genomic DNA was isolated from whole blood (QIAamp blood kit, Qiagen®) according to an established procedure.

The *ABCC6* coding region was amplified using previously described PCR primers [Wang et al., 2001]. To obtain better amplification we changed the original primer sequence for exons 15, 25, 26 and 29. In order to distinguish between *ABCC6* and its two pseudogenes, *ABCC6*-Ψ1 and *ABCC6*-Ψ2, a long-range PCR was performed of exons 1 through 10 [Pulkkinen et al., 2001]. Subsequently, PCR reactions for the separate exons were performed on the long-range amplicon. For the detection of the exon 23-29 deletion, primers described by Le Saux et al. were used [Le Saux et al., 2001].

The coding region and intron/exon boundaries of *ABCC6* were analysed with dHPLC (denaturing High Performance Liquid Chromatography) using the WAVE® System (Transgenomic® Inc., San Jose, United States) and subsequent direct sequencing of abnormal peaks using an Applied Biosystems 3100 Sequencer®, with ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems®, Foster City, United States).

All primers and dHPLC parameters are available upon request from the corresponding author. Unreported sequence variants were defined as causative using the criteria as reported by Cotton and Scriver, 1998. Nucleotide numbers are derived from cDNA *ABCC6* sequences (GenBank accession no. NM_001171.2) For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon.

RESULTS

Molecular data

The *ABCC6* gene was analysed in 38 PXE probands. Additionally, in 4 affected siblings of 3 different families, sequencing demonstrated the presence of the same mutations as those identified in the probands and as such confirms the autosomal recessive inheritance in PXE.

A total of 70 mutations were identified in 76 different disease alleles corresponding to a mutation detection rate of 92% (Table 1). Nine probands were homozygous (24%), twenty-three were compound heterozygous (60%) and in 6 patients only 1 mutation was identified. Of the 70 mutations, 36 were unique and included 3 nonsense mutations, 25 missense mutations, 6 deletions and 2 frame-shift mutations. Fourteen mutations were novel. All missense mutations altered amino acids that are highly conserved among orthologs (Mus musculus, Rattus norvegicus, Danio rerio and Fugu rubripes) and rather conserved in paralogs.

Fifty-nine of the mutations (84%) were located in the 3' half of the gene (exon 15 and beyond). Mutations were most frequently located in exons 24 (47%), mainly due to the p.R1141X mutation, 18 (8%), 28 (7%) and 29 (5%). Five new missense mutations were located in the functionally important first Nucleotide Binding Fold (NBF1) and five were located in exons coding for the second Nucleotide Binding Fold (NBF2). The nonsense mutation c.3421C>T (p.R1141X) in exon 24 was most frequently found (31/76 alleles or 41%), both in homozygous (52%) and compound heterozygous (43%) state. The Del23-29 mutation accounted for three out of seventy-six alleles (4%).

Table 1. Genotype and Phenotype of 42 Belgian PXE Patients

Patient	Sex	Age/Clinical score at initial presentation	Age/Clinical score at most recent follow-up	Mutations*			
				Allele 1		Allele 2	
01-001	F	*52* - S0, E2	*65* – S0, E3, HT	p.R1141X	c.3421C>T	**p.R760Q**	**c.2279G>A**
02-001	M	*18* – S1, E2, VR-I	*18* – S1, E2, VR-I	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
03-001	F	*59* – S1, E4	*75* – S1, E4, HT, IC, VR-I	p.R1141X	c.3421C>T	**p.N793L**	**c.2379C>G**
04-001	F	*36* – S3, E2	*36* – S3, E2	**p.N466Y**	**c.1396A>T**	p.R1339H	c.4016G>A
05-001	F	*26* – S1, E4	*43* – S3, E4, VR-I	p.R1141X	c.3421C>T	**p.T364M**	**c.1091C>T**
06-001	F	*36* – S2, E4	*44* – S2, E4, P	p.A1303P	c.3907G>C	None found	-
07-001	M	*48* – S1, E2, HT	*58* – S1, E4, HT	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
08-001	F	*26* – S1, E0	*44* – S2, E2	p.R1141X	c.3421C>T	p.R760Q	c.2279G>A
09-001	M	*49* – S0, E3, P, GIB	*65* – S2, E4, P, HT, VR-I, GIB	p.A1303P	c.3907G>C	None found	-
10-001	F	*46* – S1, E2	*63* – S3, E4, HT, AP,VR-I	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
11-001	M	*25* – S1, E2, GIB	*37* – S1, E3, GIB	p.R1141X	c.3421C>T	None found	-
12-001	F	*52* – S1, E4, CI, HT, VR-I	*52* – S1, E4, IC, HT, VR-I	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
12-002	F	*40* – S1, E2, HT, MVP, VR-I	*40* – S1, E2, HT, MVP, VR-I	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
13-001	F	*65* – S0, E2	*80* – S0, E2, P, VR-I	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
13-002	F	*57* – S3, E4	*73* – S3, E4, HT, CI, VR-I	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
14-001	F	*23* – S1, E2	*27* – S1, E2	**p.S398R**	**c.1194C>G**	**-**	**c.3364delT**
15-001	F	*27* – S1, E2	*27* – S1, E2	p.R1138W	c.3412C>T	p.R1221H	c.3662G>A
16-001	M	*51* – S2, E2	*54* – S2, E2	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
17-001	M	*42* – S1, E3, IC	*58* – S1, E3, IC	Del23-29	-	p.R518Q	c.1553G>A
18-001	M	*63* - S1, E4	*63* – S1, E4	p.E1400K	c.4198G>A	None found	-
19-001	F	*34* – S2, E2	*50* – S2, E2	p.A1303P	c.3907G>C	p.R1398X	c.4192C>T
20-001	F	*52* – S2, E2, HT, IC, GIB	*68* – S2, E4, HT, IC, GIB	p.R1141X	c.3421C>T	None found	-
21-001	M	*20* – S1, E2	*26* – S1, E2	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
22-001	M	*53* – S2, E2, IC, AP	*69* – S2, E2, HT, IC, AP	p.M751K	c.2252T>A	p.R1164Q	c.3491G>A
23-001	F	*20* – S1, E2	*27* – S1, E2, P, VR-I	**p.G666V**	**c.1996G>T**	**-**	c.1868-5T>G
24-001	M	*54* – S1, E2	*57* – S1, E2	**p.T500P**	**c.1498A>C**	**p.E521D**	**c.1563G>C**
25-001	F	*50* – S1, E3, HT, MI	*57* – S2, E3, HT, MI	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
26-001	M	*52* – S2, E4, HT	*68* – S2, E4, HT, CI	p.M751K	c.2252T>A	Del23-29	-
27-001	F	*61* – S3, E4	*68* – S3, E4, P, CI, AP	p.R1141X	c.3421C>T	-	c.4104delC

28-001	F	*31* – S1, E2	*32* – S1, E2	-	**c.1674DelC**	**p.R765W**	**c.2293C>T**
Patient	**Sex**	**Age/Clinical score at initial presentation**	**Age/Clinical score at most recent follow-up**	**Mutations*** **Allele 1**		**Allele 2**	
29-001	M	*30* – S1, E3	*32* – S1, E3	p.E125K	c.373G>A	**p.L1025P**	**c.3074T>C**
30-001	M	*65* – S0, E2, HT, CI, MI	*66* – S0, E2, HT, CI, MI	**p.G1405S**	**c.4213G>A**	None found	-
31-001	F	*38* – S1, E4	*39* – S1, E4	p.R1141X	c.3421C>T	Del23-29	-
32-001	M	*22* – S1, E2	*36* – S1, E2	p.R1141X	c.3421C>T	p.R518Q	c.1553G>A
33-001	F	*45* – S2, E3, P	*61* – S2, E3, P, VR-II	p.R1141X	c.3421C>T	p.R1141X	c.3421C>T
34-001	F	*65* – S1, E4, HT	*81* – S1, E4, HT, AP	p.R1141X	c.3421C>T	p.T1301I	c.3902C>T
35-001	F	*62* – S2, E2	*78* – S2, E2, HT	-	c.175_179del	p.G1354R	c.4060G>C
35-002	F	*58* – S2, E2	*74* – S2, E4	-	c.175_179del	p.G1354R	c.4060G>C
35-003	M	*67* – S2, E2	*79* – S2, E3, HT, VR-I	-	c.175_179del	p.G1354R	c.4060G>C
36-001	M	*53* – S1, E4	*59* – S1, E4, HT, AP	p.R1114H	c.3341G>A	p.Q1237X	c.3709C>T
37-001	M	*18* – S3, E2	*18* – S3, E2	p.Q981H	c.2943G>T	-	c.3507-3C>A
38-001	F	*27* – S1, E2	*27* – S1, E2	**p.G1263R**	**c.3787G>A**	-	c.4182delG

Table 1 represents the sex of all patients (M = male; F= female) and the age (in years – italics), respectively at initial presentation and last follow-up. Patients are identified by a code: 01 = family number; 001 = subject number. The oculocutaneous phenotype is shown as a clinical score where S = cutaneous manifestations and E = ocular manifestations. Cardiovascular and gastrointestinal complications are abbreviated as: P = weak or absent pulsations; HT = hypertension; IC = intermittent claudication; MVP = mitral valve prolapse; VR-I = mild valvular regurgitation; VR-II: severe valvular regurgitation; AP = angor pectoris; MI = myocardial infarction; GIB = gastro-intestinal bleeding. Mutations found in both alleles of *ABCC6* are shown; novel mutations are marked in bold.

* GenBank accession no. NM_001171.2. For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon.

Clinical characteristics of patients

At initial presentation, typical PXE skin and mucosal lesions, such as yellowish papules, peau d'orange or a yellow reticular pattern of the inner lip mucosa (Fig. 1A and E), were observed in 90% of all patients (38/42). All patients but two (13-001; 30-001) had characteristic microscopic skin lesions on van Giesson and von Kossa stains. The dermatological manifestations are summarized in Table 2A.

At initial presentation, all but one patient had angioid streaks (Fig. 1F). Fifty percent suffered from moderate (2/10-6/10) to severe (≤ 1/10) decrease in visual acuity. Detailed ophthalmologic findings, both at initial presentation and last consultation, are shown in Table 2B.

An increased prevalence (15%) of neurovascular manifestations (ischaemic stroke) was found in our study population (Table 3). Cardiovascular examination further revealed a high prevalence of peripheral artery disease (PAD). Fifty-three percent of all patients (22/42 – average 53 years; range 26-75 years) had abnormal Doppler examinations (atherosclerotic plaques or stenosis), most frequently of the lower extremities. Of these, 41% (9/22) had a clinical history of intermittent claudication. Cardiac ultrasound only revealed haemodynamically insignificant valvulopathies (Table 3). Only one patient had a haemodynamically significant mitral valve prolapse (MVP).

Abdominal ultrasounds revealed calcifications in kidneys, liver and/or spleen in 10 out of 17 examined patients and testicular microlithiasis in all four male patients examined [Vanakker et al., 2006]. Only three patients (7,5%) experienced gastro-intestinal bleeding. However, these were always recurrent (up to six events), requiring surgery in at least one patient (09-001).

Plasma levels of calcium and phosphorus, kidney and liver tests revealed no significant abnormalities, irrespective of either age or presence of visceral calcifications.

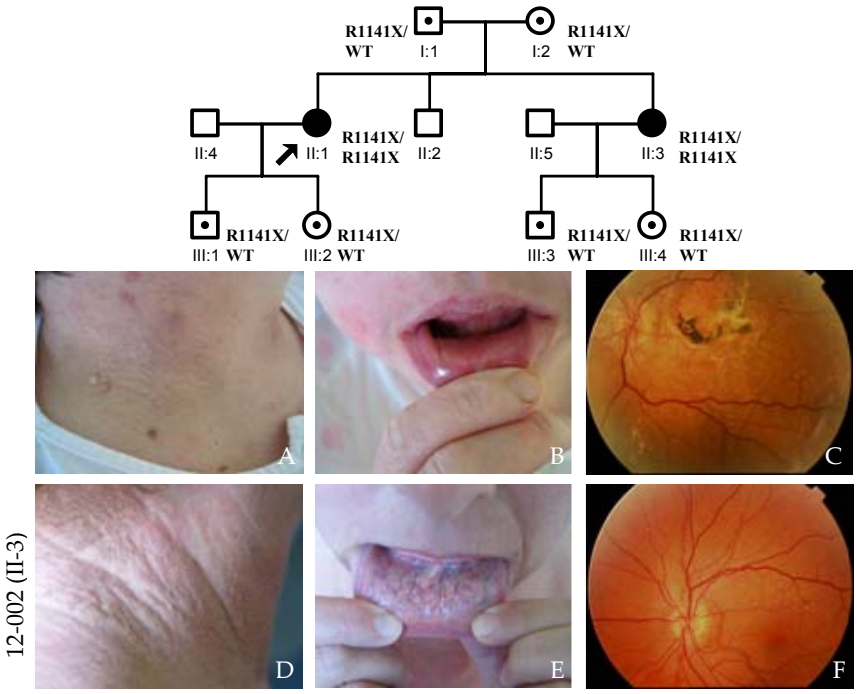

Figure 1. Oculocutaneous characteristics of family 12. The elder patient (12-001; 52 yrs.) has mild skin lesions (A-B) with characteristic histological changes, a typical PXE retinopathy with angioid streaks, subretinal bleeding and macular degeneration (C) leading to severe decrease of central vision since age 18. Patient has complaints of intermittent claudication since age 16. Her sister (12-003; 40 yrs.) has typical coalesced plaques in flexural areas of body and mucosal lesions on inner lip (D-E), a fundus with uncomplicated angioid streaks (F) and normal visual acuity. Patient has no cardiovascular complaints.

Natural history of PXE

We studied the natural history of the ocular, cutaneous and cardiovascular symptoms in a subcohort of 34 patients (ages ranged from 20 to 67 years - average age 45 years). Mean period of follow-up was 11 years (range 1-20 years).

Despite that most patients did subjectively note an enlargement of existing skin lesions, we observed progression of dermatological signs (evolving from papules to plaques or increased skin laxity) in only a limited number of patients (5/34 or 14.5%), usually starting in the fourth decade. In four of these, papular lesions coalesced into plaques (n=2) or evolved towards increased skin laxity (n=2). One patient without macroscopical skin lesions at age 49 developed plaques of coalesced papules at 65 years.

From an ophthalmologic perspective, anatomical progression (angioid streaks evolving to neovascularisation, exudation and/or haemorrhage) occurred in 9 patients (26,5%), usually starting in the sixth decade or later. However, functional decline, observed as a decrease in visual acuity was noted in 41% of patients. The mean decrease observed was 5 lines (5/10). If present, visual loss was usually binocular, albeit often consecutive. In 6 patients (17,5%), an evolution towards total loss of central vision (<1/20) ensued. Although this occurred most often in only one eye (5/6), the other eye was always severely affected (< 3/10), resulting in a considerable visual handicap.

Cardiovascular complications, most frequently hypertension, were more prominent with aging, starting from the fifth decade of life (17/25 patients or 68%). Coronary artery disease was less frequently seen in this age group (7/25). From the sixth decade on, intermittent claudication also became more apparent (8/18 patients or 44%).

Table 2. Prevalence of the Cutaneous (A) and Ocular (B) Manifestations of PXE in This Study at Initial Presentation and at Last Consultation

	Initial presentation		Last consultation	
A. Dermatological manifestations	n = 42	%	n = 42	%
No macroscopical skin lesions	4	9	3	7
Typical or mild macroscopical skin lesions present	38	90	39	93
Papular peau d'orange	35	83	35	83
Yellow reticular rash	4	9	4	9
Plaques of coalescent papules	10	24	13	31
Laxity and redundancy of skin	4	9	6	14
Buccal mucous membrane involvement	8	19	8	19
B. Ocular manifestations	n = 42	%	n = 42	%
Visual acuity				
7/10 – 10/10	21	50	18	43
2/10 – 6/10	14	33	10	24
1/10 or less	7	17	14	33
Peau d'orange	27	64	27	64
Angioid streaks	41	98	42	100
Retinal haemorrhage	5	12	10	24
Macular degeneration	11	26	16	35
Comets with or without comet-like tails	NA	NA	7	16.5

Table 2: NA: Not available.

Table 3. Prevalence of the Cardiovascular Manifestations of PXE in This Study

Cardiovascular manifestations	Patients		Carriers		GP
	n=42	%	n=17	%	%
Cerebrovascular accident	6	15	2	12	0.3-0.5[a]
(A)symptomatic PAD (abnormal Doppler result)					
Total	22	53	7	41	10-30[b]
≥55 yrs, ♂	4/10	40	5/6	34	16.9[c]
≥55 yrs, ♀	8/12	66	0	0	20.5
Hypertension	17	41	5	34	27[d]
Intermittent claudication	9	22	1	6	1-4.6[e]
Angina pectoris	5	12	1	6	
Myocardial infarction	2	5	1	6	
Mitral valve prolapse	1	2.5	0	0	
Mitral valve insufficiency					
Grade 1 – 2	11	26	8	47	90[f]
Grade 3 – 4	1	2.5	1	6	
Tricuspid valve insufficiency	16	38	0	0	17[f]
Pulmonary valve insufficiency	5	12	0	0	5[g]
Gastro-intestinal haemorrhages	3	7	0	0	

Table 3: GP = general population; [a] = [Sudlow et al., 1997]; [b] = [Criqui, 2001]; [c] = [Meijer et al., 1998]; [d] = [O'Brien et al., 2005]; [e] = [Aronow, 2005] ; [f] = [Singh et al., 1999] ; [g] = [Choong et al., 1989].

Clinical characteristics of carriers

In the carrier population (n=17) neither macroscopic skin lesions nor fundus peau d'orange or angioid streaks were observed. In two carriers, comets with comet tails were observed. Most carriers (16/17) presented no significant valvular dysfunction (Table 3). However, seven male individuals (average age 57 years) had peripheral atherosclerosis on Doppler examination, two of whom were symptomatic: one suffered from intermittent claudication at age 29 and one needed a carotid artery endarterectomy (age 56). The prevalence of (a)symptomatic PAD in European males older than 55 years is estimated to be 16.9% [Meijer et al. 1998]. Of the 6 carriers fulfilling these criteria, 5 suffered PAD (83%). Visceral calcifications were identified by abdominal ultrasound in 3 of the carriers, while 2 carrier males were found to have focal calcifications of the testicular capsule [Vanakker et al., 2006]. Blood tests showed no significant alterations.

Genotype-phenotype correlations

Table 1 summarizes the clinical manifestations of patients as a clinical score. No significant genotype-phenotype correlations emerged from this study. Especially in patients homozygous for the p.R1141X mutation – previously associated with a more severe phenotype [Hu et al., 2003]–, no significant difference with patients with either one or no nonsense mutation was observed.

DISCUSSION

Fourteen novel *ABCC6* mutations were identified in this study, bringing the total number of reported mutations at 165 [Bergen et al., 2000; Le Saux et al., 2000; Ringpfeil et al., 2000; Struk et al., 2000; Le Saux et al., 2001; Cai et al., 2001; Meloni et al., 2001; Ringpfeil et al., 2001; Le Saux et al., 2002; Hu et al., 2003b; Chassaing et al., 2004; Gheduzzi et al., 2004; Noji et al., 2004; Chassaing et al., 2005; Hendig et al., 2005; Katona et al., 2005; Miksch et al., 2005; Schulz et al. 2005 ; Schulz et al. 2005; Schulz et al. 2006; Kiéc-Wilk et al., 2007]. Exons 18, 24, 28 and 29 harbour about seventy percent of all mutations, which confirms findings of previous studies that most mutations occur either inside or downstream of exon 18. Based on these finding we propose a two-step molecular screening procedure. An initial analysis can be limited to the latter four exons (including p.R1141X) and the Del23-29 with a direct sequencing based approach. In the Belgian population, this approach would allow to detect more than 70 % of all PXE mutations. Subsequently, samples with no or only one mutation can be screened for all other exons starting at the 3' side, using direct sequencing. In our population this second step was only necessary in 50% of the PXE patients. In a minority of patients, analysis of the *ABCC6* promotor and scanning for deletions can be relevant. Such a screening strategy could be applied in other populations, provided it is adapted to the mutational spectrum of that particular population.

A large inter- and intrafamilial variability in clinical severity was noted. In some, the cutaneous manifestations were very mild or even completely absent. Others (7/40) were severely affected with increased skin laxity and redundant skin folds. A nice example of intrafamilial variability is shown in Figure 1. Our observations suggest that the type of skin lesion usually remains identical throughout the evolution of the disorder. In a minority of PXE patients, skin lesions evolve towards large coalesced plaques of papules and/or moderate to severe laxity of the skin. Especially the latter can cause considerable esthetical and psychological problems. On the other hand, three patients (7%) had no apparent macroscopic skin lesions. As such, a negative skin inspection can not exclude the diagnosis of PXE.

The presence of angioid streaks in all patients in this study is not surprising, since ours is a somewhat older population. In our experience all PXE patients develop angioid streaks in their second or third decade. Therefore we feel that at the age of 30 years, angioid streaks are mandatory to make a diagnosis of PXE. In younger individuals, streaks are often preceded by peau d'orange which should suffice as an ophthalmologic sign [Neldner and Struk, 2002; Hu et al., 2003]. The fact that only 64% of our patients had peau d'orange fundus lesions is also related to the high average age of our study population, since peau d'orange regresses with age [Neldner and Struk, 2002]. Comets and comet tails have been described as a pathognomonic and early sign of PXE [Gass, 2003; Wegman et al., 2005]. We found these white, punched-out fundus lesions in some of the patients (7/42) but, more surprisingly, also in carriers (2/17). Given the presence of disseminated foci of visceral and testicular hypercalcification of both patients and carriers [Vanakker et al., 2006], the comets and comet tails may equally represent areas of hypercalcification of the Bruch membrane and chorioretina. This hypothesis is indirectly

substantiated by the concurrent presence of abdominal and/or testicular calcifications in 5 out of 6 patients and 1 out of 2 carriers who underwent extensive systemic ultrasound evaluation, and presented with comets and/or comet tails in the fundus.

Cerebrovascular manifestations were remarkable in that there was a considerably higher prevalence of ischaemic stroke in our patient cohort (6/42 or 15%) than in the general population (0.3-0.5%) [Sudlow and Warlop, 1997]. Our observations confirm the association between PXE and stroke described in several case reports [McKusick, 1966; Neldner, 1988; Schievink et al., 1994; van den Berg et al., 2000]. It usually does not occur until the fifth or sixth decade in PXE [Pieczuro et al., 1981; Schievink et al., 1994].

Further cardiovascular work-up revealed an increased incidence of PAD on Doppler examination. However, 59% of these were completely asymptomatic. In the latter, subclinical atherosclerotic plaques were mostly detected in the femoral arteries. This suggests that in PXE patients, Doppler evaluation of the vessels in the lower extremities may be equally important as that of the carotid arteries. Identification of a significant MVP in only one patient in this study reflects the overdiagnosis of prolapse before revision of valvular anatomy and diagnostic criteria in the late 1980s [Hayek et al., 2005]. Although often severe and recurrent, gastro-intestinal haemorrhages only occurred in 3 patients, which suggests that this is a less frequent complication than reported previously [Neldner, 1988]. However, since gastro-intestinal haemorrhaging is a serious complication, secondary prevention after stroke with anti-platelet drugs poses a therapeutic dilemma [Aessopos et al., 1997].

Some of the findings in carriers were also unusual (Table 3). The increased incidence of (a)symptomatic PAD, suggests that heterozygosity for an *ABCC6* mutation may be a risk factor for common diseases such as atherosclerosis or hypertension. The presence of small calcifications in the abdominal organs and testicles, as well as comets and comet tails in fundo subscribes to the hypothesis of subclinical manifestations in carriers. They are however, most probably of little clinical significance.

In light of the current clinical and molecular knowledge, we have attempted to develop a diagnostic flowchart, defining the role of skin biopsy and molecular analysis within a diagnostic work-up of a patient thought to suffer from PXE (Fig. 2). Our initial inclusion criteria (typical ophthalmological and skin manifestations, a positive skin biopsy) were very strict and derived from the criteria of Lebwohl et al., 1994. In clinic, patients either present first with cutaneous or with ocular manifestations. Of these, cutaneous changes are most often the first sign noticed by either patient or physician. Indeed, visual complaints only occur later during the course of the disease, as a complication of subretinal neovascularisation. In order to make a clinical diagnosis of PXE the combination of dermatological inspection and fundoscopy remains essential.

The clinical diagnosis is certain when both skin (papular lesions, plaques) and fundus findings (peau d'orange, angioid streaks) are typical of PXE, provided that the phenocopy associated with haemoglobinopathies has been excluded in Mediterranean and African countries [Aessopos et al., 1997]. In these cases, *ABCC6* analysis may confirm the clinical diagnosis, which may obliviate the need for an initial skin biopsy.

ABCC6 analysis can also be useful when either the skin or fundus lesions are the only clinical findings. Even in absence of a positive family history, PXE remains the most probable diagnosis in cases where typical skin or fundus manifestations are present, making the use of molecular testing an efficient alternative for skin biopsy or an added value for screening the family. A skin biopsy remains essential in cases where clinical evidence for PXE is very limited (normal fundus and very mild papules).

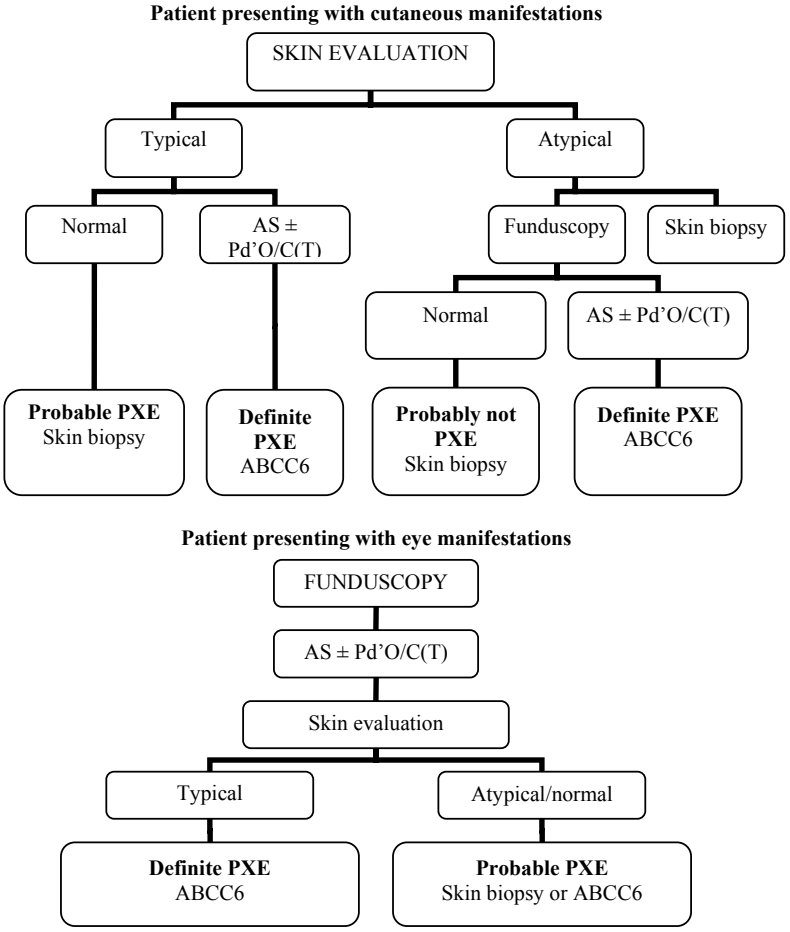

Figure 2. Diagnostic flowchart for Pseudoxanthoma elasticum. AS = angioid streaks; Pd'O= peau d'orange; C(T) = comets or comet tails; FH = family history; typical skin lesions = yellowish papules, peau d'orange, yellow reticular pattern of lip; mild skin lesions = papular or reticular pattern suggestive of PXE. "ABCC6" = molecular analysis of the *ABCC6* gene. Based on the clinical findings during skin and fundus evaluation, a diagnosis of PXE can either be definite or probable. A positive family history can in some cases add to degree of certainty of diagnosis. When having definitive clinical diagnosis of PXE, *ABCC6* analysis is sufficient for confirmation.

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Publication 2

Mutation detection in the *ABCC6* gene and genotype-phenotype analysis in a large international case series affected by pseudoxanthoma elasticum.

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This study arose from an international joint effort of PXE International – the US patient advocacy group – together with the Ghent group and the South African PXE group. It aimed to evaluate the efficacy of molecular analysis of the *ABCC6* gene and genotype-phenotype correlations in a large –so far the largest – case series of PXE patients.

ABCC6 was analysed using dHPLC and direct sequencing in 270 patients (239 probands, 31 affected family members), of which clinical data was obtained using a detailed questionnaire.

Not only does this study contribute to the description and expansion of the *ABCC6* mutation spectrum with 39 novel mutations, but – due to its magnitude – unambiguously demonstrates that no distinct clinical correlate can be delineated from the patients' mutations. It emphasizes that phenotypic variability in PXE must be due to other mechanisms besides *ABCC6* mutation characteristics (type, location or functional effect), such as modifier genes or epigenetic modifications.

ORIGINAL ARTICLE

Mutation detection in the *ABCC6* gene and genotype–phenotype analysis in a large international case series affected by pseudoxanthoma elasticum

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Background: Pseudoxanthoma elasticum (PXE), an autosomal recessive disorder with considerable phenotypic variability, mainly affects the eyes, skin and cardiovascular system, characterised by dystrophic mineralization of connective tissues. It is caused by mutations in the *ABCC6* (ATP binding cassette family C member 6) gene, which encodes MRP6 (multidrug resistance-associated protein 6).
Objective: To investigate the mutation spectrum of *ABCC6* and possible genotype–phenotype correlations.
Methods: Mutation data were collected on an international case series of 270 patients with PXE (239 probands, 31 affected family members). A denaturing high-performance liquid chromatography-based assay was developed to screen for mutations in all 31 exons, eliminating pseudogene coamplification. In 134 patients with a known phenotype and both mutations identified, genotype–phenotype correlations were assessed.
Results: In total, 316 mutant alleles in *ABCC6*, including 39 novel mutations, were identified in 239 probands. Mutations were found to cluster in exons 24 and 28, corresponding to the second nucleotide-binding fold and the last intracellular domain of the protein. Together with the recurrent R1141X and del23–29 mutations, these mutations accounted for 71.5% of the total individual mutations identified. Genotype–phenotype analysis failed to reveal a significant correlation between the types of mutations identified or their predicted effect on the expression of the protein and the age of onset and severity of the disease.
Conclusions: This study emphasises the principal role of *ABCC6* mutations in the pathogenesis of PXE, but the reasons for phenotypic variability remain to be explored.

Pseudoxanthoma elasticum (PXE) is an inherited disorder in which mutations in the *ABCC6* (ATP-binding cassette family C member 6) gene lead to dystrophic mineralization and fragmentation of connective tissues.[1–4] Multiple organs are affected, including the skin, eyes and cardiovascular system, and the pathogenic changes include lax and inelastic skin, angioid streaks in the retina and mineralization of the internal elastic lamina of midsized arteries, including the cerebral, coronary, gastrointestinal and peripheral vasculature. Onset is often in late childhood or adolescence, when yellowish cutaneous papules may be noted, most commonly on the neck, axillae and antecubital fossae. However, in many cases angioid streaks in the ocular fundus are the initial physical finding, which correspond to breaks in the elastin-rich Bruch's membrane of the choroid. As the disease progresses, fragile new vessels may grow through the angioid streaks and hemorrhage, leading to central vision loss. The cardiovascular system may also be affected, leading to hypertension, intermittent claudication, gastrointestinal bleeding, ministrokes and rarely, myocardial infarction. Early identification of the disease and increased surveillance for its sequelae might improve the quality and length of life of those affected. Not only would early detection of mutations in the *ABCC6* gene in at-risk people facilitate diagnosis, but elucidation of possible genotype–phenotype correlations might enable the prediction of disease severity and development of early intervention strategies.

Inheritance of PXE is autosomal recessive; although a few families have been reported in which two generations are affected, pathogenetic mutations in both *ABCC6* alleles of affected people were present indicating pseudodominance.[2 5 6]

Mineralization and fragmentation of elastic fibers, the hallmark of PXE, results from altered function of multidrug resistance-associated protein 6 (MRP6), the gene product encoded by *ABCC6*. The *ABCC6* gene is located at chromosome 16p13.1[7 8] along with two closely related but nonfunctional 5′ pseudogenes, *ABCC6-Ψ1* and *ABCC6-Ψ2*, corresponding to exons 1–9 and 1–4, respectively.[9] The close sequence similarity of the two pseudogenes to the actual coding gene complicates mutation detection and sequencing, although coding gene-specific primer sets can be designed for each exon without interference from the pseudogene sequences.[9] The *ABCC6* gene is encoded in 31 exons spanning approximately 75 kb of the human genome and is transcribed into an mRNA of~5 kb and translated into a 165 kDa protein of 1503 amino acids.[7] It is expressed primarily in the liver and functions as a putative efflux transporter of currently unknown substrate specificity, although recent studies have shown that it can function as a transmembrane transporter of polyanionic, glutathione conjugated molecules in vitro.[10–13] In addition to high levels of *ABCC6* expression in the liver, clearly measurable levels of

Abbreviations: ABCC6, ATP-binding cassette family C member 6; dHPLC, denaturing high-performance liquid chromatography; IC, intracellular domain; MRP6, multidrug resistance-associated protein 6; NBF, nucleotide-binding fold; PTC, premature termination codon; PXE, pseudoxanthoma elasticum; TM, transmembrane domain

expression are detected in the proximal tubules of kidneys, and lower, barely detectable levels are found in the skin, retina and blood vessels, tissues most severely affected by PXE.[14–16]

MRP6 has three hydrophobic membrane-spanning domains containing a total of 17 transmembrane helices and two intracellular nucleotide-binding folds (NBFs), comprising 3 highly conserved Walker motifs, and is critical for the putative function of the protein in transmembrane transport driven by the energy from ATP hydrolysis. To date, >150 distinct mutations in the *ABCC6* gene have been described in the literature including missense and nonsense mutations in 27 of the 31 exons and deletions spanning the entire coding region.[2 15 17–38] Clustering of missense mutations to exons corresponding to the nucleotide-binding folds and a few intracellular segments connecting two transmembrane domains have been noted. Two recurrent mutations, R1141X and a large deletion of exons 23–29 (del23–29, p.A999_S1403del) have been described in a significant proportion of patients, leading to a mutation-detection strategy that first identifies the recurrent mutations by restriction-enzyme digestion, followed by sequencing of the remaining exons.[39]

In this study, we collected genotype data on 270 patients with PXE from 239 families and were able to characterise the phenotype in 198 of these patients to improve diagnosis, identify genotype–phenotype associations and facilitate genetic counselling of people at risk for PXE.

Table 1 Phenodex assignment of patients with PXE to different phenotypic categories based on clinical findings in five organ systems

System	Findings
Skin	
S0	No sign
S1	Papules/bumps
S2	Plaques of coalesced papules
S3	Lax and redundant skin
Eye	
E0	No sign
E1	Peau d'orange
E2	Angioid streaks
E3	Bleeding and/or scarring
GI	
G0	No sign
G1	GI bleeding as related to PXE
Vascular	
V0	No sign
V1	Weak or absent pulses
V2	Intermittent claudication
V3	Vascular surgery
Cardiac	
C0	No sign
C1	Chest pain/angina/abnormal ECG or abnormal stress test with no symptoms
C2	Heart attack

ECG, electrocardiogram; GI, gastrointestinal.

METHODS

Informed consent was obtained from all patients and the study was approved by the institutional review board of Good Samaritan Medical Center, Brockton, Massachusetts and the Genetic Alliance BioBank Institutional Review Board.

Donors and samples

Blood samples were collected from 270 patients with PXE (239 probands and 31 affected family members) from the USA, Canada, Europe, Australia and South Africa. None of these families was previously used in other mutation analysis datasets. A detailed questionnaire was used, covering demographic information, family history, and dermatological, ophthalmological, gastrointestinal, cardiac, vascular, neurological, orthopaedic, gynaecological and nutritional histories and physical findings, which was used to ascertain the phenotype of the patients for genotype–phenotype correlations. The phenotypic characteristics were summarised according to organ system and severity (Phenodex PXE International; see table 1). In all index cases, the diagnosis of PXE was made according to the consensus criteria of Lebwohl *et al*[3] and confirmed by skin biopsy specimens stained with H&E and/or von Kossa stain. People with a positive family history (ie confirmed PXE in a first-degree relative and either eye or skin signs), were also considered to be affected.

Mutation detection

Genomic DNA was isolated from peripheral blood samples (Puregene DNA Isolation Kit; Gentra Systems, Minneapolis, Minnesota, USA). Control genomic DNA was obtained from the human lymphoblastoid cell line K562 (American Type Culture Collection, Manassas, Virginia, USA). All DNA samples were adjusted with water to a concentration of 10 ng/μL.

Our mutation-detection strategy was based on: (1) identification of the recurrent mutations R1141X and del23–29 by restriction-enzyme digestion; (2) optimised denaturing high-performance liquid chromatography (dHPLC) scanning of PCR products corresponding to all exons in subjects in whom the two recurrent mutations were not identified on both alleles, followed by (3) sequencing of exons with altered dHPLC patterns; and (4) confirmation of novel mutations by restriction-enzyme digestion or resequencing.

Screening for the recurrent mutations R1141X and del23–29 was performed as previously described.[30 31] Conditions and primers for generating PCR products spanning all exons of the coding regions and flanking intronic sequences of the *ABCC6* gene were identified for optimum dHPLC screening (supplementary table 1; available at http://jmg.bmj.com/supplemental). These primers were designed to exclude the pseudogenes homologous to exons 1–4 and 1–9[31] and to anneal within ~50 bases of the 5′ and 3′ ends of the exon and to exclude known intronic polymorphisms where possible. In many cases, the primers originally designed for exons 10–31 were not suitable for sequencing, and new primers corresponding to sequences situated >50 bases from the intron–exon boundaries were designed to improve sequencing results. PCR for dHPLC analysis was performed using 1.5 U Taq polymerase (Qiagen Inc., Valencia, California, USA) mixed with 5 U Optimase Taq polymerase (Transgenomic, Gaithersburg, Maryland, USA) and Q buffer (Qiagen), according to the manufacturer' instructions. PCR reactions contained 200 ng DNA as template and 20 ng of each primer in a final volume of 50 μl. Cycling conditions for all primer pairs were 94℃ for 5 min, followed by 41 cycles of 94℃ for 1 min, annealing temperature for a particular primer pair (range 55–60℃) for 1 min and 72℃ for 1 min, with a final step at 72℃ for 5 min.

The PCR products generated using patients with PXE DNA as template were allowed to form heteroduplexes with an equal volume of a PCR product of the same exon amplified from template DNA of the lymphoblastoid control cell line K562. For this purpose, the PCR products were mixed in a 1:1 ratio and denatured at 94℃ for 10 min, followed by reannealing at 65℃ for 15 min and 37℃ for 15 min. The PCR products were screened by dHPLC (WAVE; Transgenomic, Gaithersburg, Maryland, USA) using methods designed to enhance partial denaturation of the PCR products containing mismatched bases (supplementary table 1; available at http://jmg.bmj.com/supplemental). PCR products showing pattern shifts were sequenced in both directions in most cases. DNA sequencing was performed on an automated sequencer (ABI Prism 377 or ABI

3100; Perkin-Elmer-Cetus, Foster City, California, USA). Putative mutations were confirmed by restriction-enzyme digestion followed by agarose-gel electrophoresis or by resequencing of a new PCR product when a suitable restriction enzyme was not available. Novel amino acid substitution mutations that affected conserved residues and were not found in 200 control alleles were considered causal.[40]

Genotype–phenotype correlation analyses

Only patients who filled out the complete clinical questionnaire and in whom the mutations in both alleles had been characterised were included in the genotype–phenotype correlation analysis. Of an original cohort of 270 patients, 134 fulfilled both these criteria. These were 94% Caucasian (all of European descent: 80% USA, 8% Canada, 7% South Africa, 2% UK, 2% Australia, 1% other European countries), 4% Native Americans, 1% Hispanic, 0.5% African American and 0.5% Pacific Islander. Medical records for >10% of the respondents were compared with the questionnaire responses to determine whether the results were representative. Two epidemiologists (SH, MM) analysed the data, using SAS® version 9.1 (Cary, NC).

For the purposes of the genotype–phenotype correlation study, the symptoms and signs were scored as presented in table 1. The data gathered indicated that PXE primarily affected five main clinical areas: skin (S), eyes (E), gastrointestinal system (G), heart (C) and vasculature (V). The data also indicated that for each of these areas there were between two and four grades of severity.

The genotype–phenotype analysis consisted of two parts. First, subjects were grouped by the probable effect of their mutation on protein function: (1) no functional protein, including premature termination codon causing mutations and out-of-frame insertions and deletions; (2) some functional protein, such as in-frame deletions or insertions and missense mutations; and (3) those where it was not possible to predict whether functional protein would be made, such as splicing mutations.

In the second part of the analysis, subjects with PXE were grouped according to the location of their mutations along the putative MRP6 protein (ie, intracellular domains, transmembrane domains, or other).

For each approach, groups were compared using the Fisher exact test.

RESULTS

Mutation detection

Mutation detection was performed on 270 samples from 239 families. The mean (SD) age of the patients was 45 (15.9) years (range 3–81) and 72% were female (n = 173). Of the 239 index cases, 31 (31/240, 12.9%) had no identifiable mutations. Collectively, 316 mutant alleles were identified, yielding an overall detection rate of 66% (316/478) (all mutations and combinations are listed in supplementary table 2; available at http://jmg.bmj.com/supplemental).

In total, 82 distinct mutations were identified in this case series (table 2). The two most common mutations were R1141X (29.3%, 92/316) and del23–29 (18%, 57/316). In all, 23 subjects (9.6%) were homozygous for either the R1141X (n = 11) or del23–29 (n = 2) or compound heterozygous (n = 10) for these two mutations. All other mutations were found in <10 subjects (table 2). A total of 51 mutations occurred only once. Two subjects carried three mutations, with two PXE mutations on one allele, although haplotype phase could not be determined because of lack of parental samples. No mutations were identified in 161 alleles and there was an insufficient quantity of one patient's DNA for complete genotyping.

Further dHPLC screening of the *ABCC6* gene resulted in the identification of recurrent but less common mutations. Of these, R1339C, R1164X and 2787+1g→c represented 5.0%, 4.7% and 2.8%, respectively, of the 316 alleles identified. R1339C was common in the South African Afrikaner population (15 of 40 alleles, 37.5%) and rare in the European/American case series (1/238 alleles). The high incidence of R1339C in the South African population is probably due to a founder effect.[26] Conversely, R1164X (0/40 alleles) and 2787+1g→c (0 of 40 alleles) were absent in the South African case series but were prevalent in the European and American patient populations.

Novel mutations

In all, 38 novel, previously unreported mutations were identified in 61 patients in this study (table 2, figs 1 and 2). Most were missense mutations (23 of 39), but there were also four splicing mutations, four insertions, three deletions and five nonsense mutations that had not been previously reported.

Prevalence of mutations by exon

Assignment of all of the mutations in this multi-national case series by exon showed that most point mutations occurred in exons 24 (133/316, 42%) and 28 (36/316, 11%) with a smaller number in exons 9 (14/316, 4.4%) and 18 (10/316, 3%) of the *ABCC6* gene (table 2, fig 1). Collectively, the mutations in exons 24 and 28, including the common mutations R1141X and del 23–29, accounted for 71.5% of all the 316 mutations identified in this study (table 2), and the 11 most prevalent mutations (R1141X, del23–29, R1339C, R1164X, 2787+1G→T, G1302R, R1138Q, R1138W, Q378X, R1314W, R518Q) accounted for 70% (223 of 316) of the mutant alleles identified (table 2).

Missense mutations cluster in certain domains

Of the 82 distinct mutations detected in this study, 48 (48/82, 58.5%) were missense mutations and were found to cluster in certain domains of the MRP6 protein (table 2, fig 2). The NBFs were found to harbour a large proportion of missense mutations (NBF1 10/49, 20.4%, NBF2 10/49, 20.4%), apparently reflecting the biological importance of these regions in the binding and hydrolysis of ATP and their critical role in the function of the MRP6 protein. Similarly, other cytoplasmic, intracellular domains (IC) were found to harbour a relatively large proportion of missense mutations (19/49, 38.7%) but these clustered in a number of domains, with the majority found in IC8 (7/49, 14.2%), again reflecting the potential importance of this domain in terms of the function of the protein. Notably, certain transmembrane domains (TM) were completely devoid of missense mutations (TM 3–5, 8, 14–17).

Phenotyping of index cases

Of the 197 patients with full phenotypic data, the characteristics were distributed as follows: 193 people (98%) had some skin signs, 176 (89%) had eye signs, 136 (69%) had vascular signs or symptoms, 50 (25%) had cardiac signs or symptoms and 16 (8%) had gastrointestinal signs (supplementary table 2; available at http://jmg.bmj.com/supplemental). To examine these distributions by age, these patients were categorised into three age categories: (1) <40, (2) 40–54 and (3) >55 years. The mean skin score was 2.2 in all three age categories, the mean scores for eye, vascular and cardiac symptoms increased with age, and the mean gastrointestinal score was nearly 0 in those aged <40 and was 0.1 in the other two age categories.

Genotype–phenotype correlations

The comparison of subjects whose mutations would probably have resulted in no functional protein with those whose mutations would probably have resulted in some functional

Table 2 Distinct mutations identified in the international case series of 271 patients with PXE

Nucleotide change*†	Predicted consequence†	Frequency (alleles)	Exon–intron location	Domain affected‡	Mutant alleles (%)	References§
c.105delA	p.S37fsX80	2	2		0.6	28
c.177–185del9	p.R60_Y62del	1	2		0.3	9, 28
c.179del12ins3	**p. R60_W64del L60_R61ins**	1	2		0.3	
c.220-1g→c	**SJ**	1	IVS 2		0.3	
c.724g→t	**p.E242X**	1	7		0.3	
c.938insT	FS	1	8		0.3	25
c.998+2delT	SJ	1	IVS 8		0.3	2, 21
c.998+2del2	SJ	1	IVS 8		0.3	18
c.951c→g	p.S317R	2	9	TM6	0.6	28
c.1087c→t	**p.Q363X**	1	9		0.3	
c.1091g→a	p.T364R	1	9	TM7	0.3	9, 19, 21, 28
c.1132c→t	p.Q378X	4	9		1.2	9, 17–19, 28, 37
c.1144c→t	**p.R382W**	2	9	IC4	0.6	
c.1171a→g	p.R391G	3	9	IC4	0.9	9, 18, 28, 37
c.1176g→c	**p.K392N**	1	9	IC4	0.3	
c.1388t→a	**p.L463H**	1	11	TM9	0.3	
c.1484t→a	p.L495H	1	12	IC5	0.3	28
c.1552c→t	p.R518X	2	12		0.6	18, 19, 27, 28, 37
c.1553g→a	p.R518Q	4	12	IC5	1.2	18, 19, 24, 28, 31
c.1603t→c	**p.S535P**	1	12	TM10	0.3	
c.1703t→c	p.F568S	1	13	TM11	0.3	24
c.1798c→t	**p.R600C**	1	14	TM11	0.3	
c.1857insC	FS	1	14		0.3	
c.1987g→t	**p.G663C**	1	16	NBF1	0.3	
c.1999delG	FS	1	16		0.3	
c.2070+5G→A	SJ	2	IVS 16		0.6	
c.2093a→c	**p.Q698P**	2	17	NBF1	0.6	
c.2097g→t	**p.E699D**	1	17	NBF1	0.3	
c.2177t→c	**p.L726P**	1	17	NBF1	0.3	
c.2237ins10	FS	2	17		0.6	
c.2252t→a	p.M751K	1	18	NBF1	0.3	20, 37
c.2263g→a	**p.G755R**	2	18	NBF1	0.6	
c.2278c→t	p.R760W	3	18	NBF1	0.9	20, 28, 32, 37
c.2294g→a	p.R765Q	2	18	NBF1	0.6	20-22, 25, 28, 32, 37
c.2329g→a	**p.D777N**	1	18	NBF1	0.3	
c.2359g→t	**p.V787I**	1	18	NBF1	0.3	
c.2432c→t	p.T811M	1	19	IC6	0.3	6
c.2643g→t	**p.R881S**	1	20	IC6	0.3	
c.2787+1G→T	SJ	9	IVS 21		2.8	17, 20, 24, 28, 31, 37
c.2814c→g	**p.Y938X**	1	22		0.3	
c.2820insC	**FS**	1	22		0.3	
c.2831c→t	**p.T944I**	1	22	TM12	0.3	
c.2848g→a	**p.A950T**	1	22	TM12	0.3	
c.2974g→c	p.G992R	1	22	TM13	0.3	2, 42
c.3340c→t	p.R1114C	2	24	IC8	0.6	19, 28, 32, 37, 41
c.3389c→t	p.T1130M	3	24	IC8	0.9	18, 19, 21, 22, 28, 30, 32, 37, 41
c.3398g→c	**p.G1133A**	1	24	IC8	0.3	
c.3412g→a	p.R1138W	7	24	IC8	2.2	28, 30, 37
c.3413c→t	p.R1138Q	7	24	IC8	2.2	18, 19, 24, 25, 28, 30, 32, 37, 41
c.3415g→a	**p.A1139T**	2	24	IC8	0.6	
c.3415g→a & c.2070+5G→A*	**p.A1139T & SJ**	1	24, IVS 16	IC8	0.3	
c.3415g→a & c.4335delG*	**p.A1139T & FS**	1	24, 30	IC8	0.3	
c.3421c→t	p.R1141X	92	24		29.3	5, 9, 15,18, 19, 21, 22, 24, 28, 30–32, 33, 37, 41
c.3427c→t	**p.Q1143X**	1	24		0.3	
c.3490c→t	p.R1164X	15	24		4.7	18, 27, 28, 31, 33
c.3491g→a	p.R1164Q	1	24	IC8	0.3	28
c.3661c→t	p.R1221C	1	26	IC9	0.3	21, 22, 28, 29
c.3662g→a	p.R1221H	2	26	IC9	0.6	40
c.3676c→a	**p.L1226I**	1	26	IC9	0.3	
c.3722g→a	**p.W1241X**	2	26		0.6	
c.3774insC	**FS**	2	27		0.6	
c.3775delT	p.G1259fsX1272	3	27		0.9	15, 25, 28, 41
c.3880-3882del	**p.K1294del**	1	27		0.3	
c.3883-5G→A	**SJ**	1	IVS 27		0.3	
c.3892g→t	p.V1298F	1	28	NBF2	0.3	25
c.3904g→a	p.G1302R	7	28	NBF2	2.2	21, 22, 25, 28
c.3907g→c	p.A1303P	1	28	NBF2	0.3	21, 22, 25, 28
c.3912delG	FS	1	28		0.3	28
c.3940c→t	p.R1314W	4	28	NBF2	1.2	24, 25, 32, 36
c.3941g→a	p.R1314Q	1	28	NBF2	0.3	25, 28, 32, 36, 41
c.4004t→a	**p.L1335Q**	1	28	NBF2	0.3	
c.4015c→t	p.R1339C	16	28	NBF2	5.0	19, 25, 28, 33
c.4016g→a	**p.R1339H**	2	28	NBF2	0.6	
c.4025t→c	**p.I1342T**	1	28	NBF2	0.3	

Table 2 Continued

Nucleotide change*†	Predicted consequence†	Frequency (alleles)	Exon–intron location	Domain affected‡	Mutant alleles (%)	References§
c.4041g→c	p.Q1347H	1	28	NBF2	0.3	25
c.4104delC	FS	1	29		0.3	25
c.4192c→t	p.R1398X	2	29		0.6	25
c.4335delG	**FS**	1	30		0.3	
c.4441g→a	**p.G1481S**	1	31	NBF2	0.3	
c.4501g→a	**p.G1501S**	1	31	NBF2	0.3	
Ex23_29del	p.A999_S1403del	57	23–29		18.0	15, 18, 21, 25, 27, 28, 31, 32, 37, 44, 45

FS, frameshift; IC, intracellular domain; IVS, intron; NBF, nucleotide binding fold; SJ, splice junction; TM, transmembrane domain.
*The numbering system corresponds to ABCC6 cDNA (GenBank accession number NM00171.2), the adenosine residue in translation initiation codon ATG being +1. The combination of mutations indicated by an asterisk were identified on one allele of the same patient.
†Mutations in bold are novel.
‡In cases of missense mutations, the affected protein domain is indicated.
§In cases of previously published mutations, the corresponding references are listed.

protein did not yield significant differences. Secondly, subjects with PXE and with missense mutations on one or both alleles were grouped according to the location of their mutation (NBF, IC, TM). Each group was compared with the other three groups but no significant differences were obtained (data not shown). Subjects who had mutations in two different locations (n = 8) were assigned initially to the first location, and the analysis then repeated with these subjects assigned to the second mutation location. Again, no significant differences were found in these analyses.

Although there is no evidence to support the assumption that protein function can be revealed by type of mutation in the absence of functional data, we attempted to confirm the finding of Schulz *et al*,[32] that the age of PXE diagnosis and the number of organ systems affected is significantly different between patients with predicted non-functional protein compared with those with some potentially functional protein. The classifications were made according to predicted consequences to the protein; missense mutations were predicted to result in some protein that may be functional, whereas nonsense, insertion and deletion mutations were predicted to result in non-functional protein. The consequence of splicing mutations could not be predicted. Unfortunately, the number of patients in whom a definite age of diagnosis was known was limited to 50 patients. The mean age at diagnosis of patients with a non-functional protein in this study was 28.5 (n = 19), and that of patients with some protein function was 28.8 (n = 31). These differences were not significant (p = 0.95). When the analysis was repeated for reported age of onset of symptoms regardless of age of diagnosis, presuming that age at onset is younger than age at diagnosis, again no significant differences were found.

DISCUSSION

This study identified a number of novel and recurrent mutations in the *ABCC6* gene in a large multinational case series of patients with PXE. In all, 82 distinct mutations, of which 39 were novel, previously unreported, were identified in 239 probands. The detection rate of 66% is less than that reported by other groups, and potentially reflects (1) the lower detection rate when using dHPLC for screening,[41 42] (2) the inability of the methods used to detect large deletions including heterozygous loss of entire exons or the entire *ABCC6* gene, and (3) presence of mutations in regions such as the 5′ regulatory elements, 3′ untranslated region and central intronic sequences of *ABCC6*, which were not analysed in this study. Finally, there is the possibility that mutations in genes other than *ABCC6* can

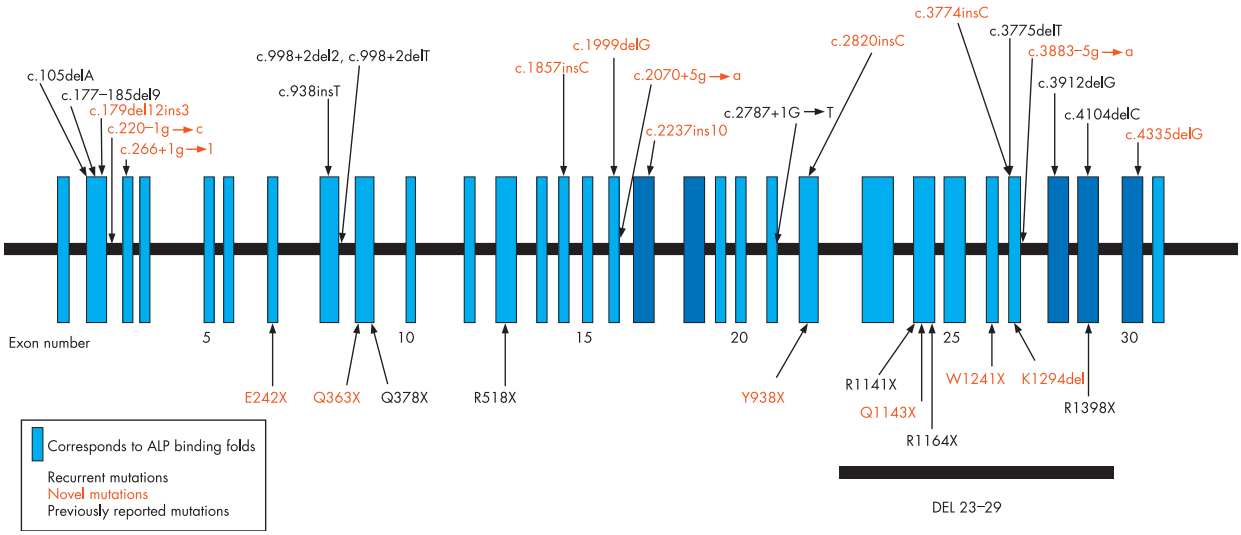

Figure 1 The positions of nonsense, splice junction, insertion and deletion mutations identified in the *ABCC6* gene in patients with pseudoxanthoma elasticum (PXE). Vertical blue boxes represent the 31 exons, and every fifth exon is numbered. Splicing, small insertion and deletion mutations are shown above the line, and nonsense mutations below, with the position of the recurrent del23–29 mutation. Dark blue boxes, nucleotide-binding fold domains; bold, recurrent mutations; red, novel mutations.

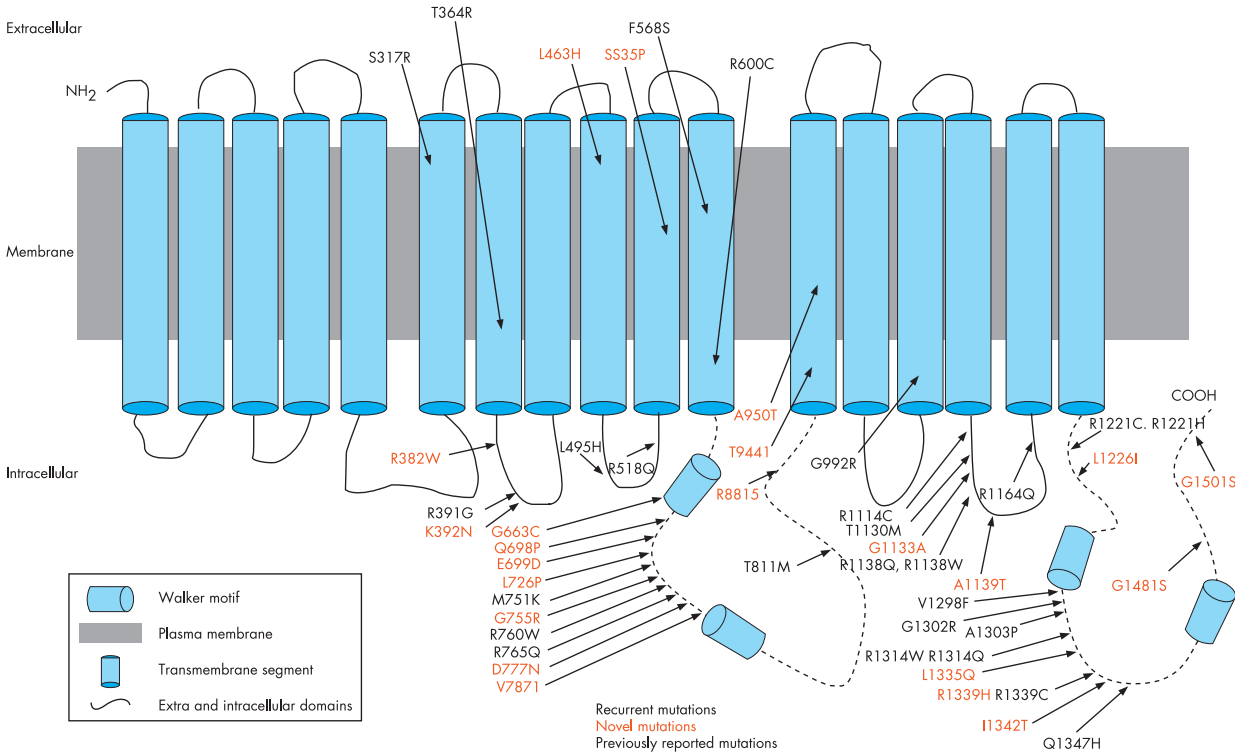

Figure 2 Schematic representation of the MRP6 protein and the positions of missense mutations identified in patients with pseudoxanthoma elasticum (PXE). Vertical blocks are the 17 transmembrane domains of the MRP6 protein; dotted lines, nucleotide-binding fold; bold, recurrent missense mutations; red, novel mutations.

result in a PXE phenotype, although no concrete evidence for this possibility currently exists. The recurrent mutations R1141X and del23–29 were the most prevalent, consistent with previous reports. In our international case series, mutations in exons 24 and 28 together with del23–29 accounted for the majority of the mutations detected (71.5%) in the *ABCC6* gene. Several other recurrent mutations were also identified and comprised around 20% of the total number of mutations.

The NBF domains of the MRP6 protein harboured a large number of missense mutations (22/49), reflecting the strong amino acid sequence conservation in this region, necessary to maintain the function of the protein (fig 2). In addition, intracellular domain 8 harboured several missense mutations (8/49), suggesting the importance of sequence conservation in this region of the protein and perhaps a function that has yet to be identified.

Genotype–phenotype analysis of the 134 patients for whom both *ABCC6* mutations and full phenotypic data were available failed to reveal any definitive correlations. The correlation of age of PXE diagnosis with the predicted consequence of the mutation (protein or no protein), as reported by Schulz *et al*,[32] could not be confirmed. As there is no empirical confirmation of whether there is functional MRP6 protein as a result of each of the mutations, the accuracy of this conclusion rests on the validity of the presumption that the mutation either does or does not lead to functional MRP6 protein. Molecular studies of three different MRP6 missense mutations (V1298F, G1302R and G1321S) performed in vitro found that none of these three mutations resulted in ATP-dependent substrate transport although ATP binding was normal.[11] Although these in vitro data do not address whether the proteins bearing these missense mutations are actually formed in the cell and inserted properly into the membrane, it confirms the hypothesis that a missense mutation in a critical portion of the molecule can completely ablate the function of the protein and result in a phenotype as severe as if no protein was present at all.

Finding no genotype–phenotype correlations in complex mendelian disorders, such as metabolic diseases, is not uncommon.[43 44] This lack of association suggests that no simple relationship exists between the type and position of mutations, the mutations themselves and the severity of disease. Thus, it is not possible from these data to predict the type or severity of phenotypic manifestations from mutation studies themselves. Because the type of phenotypic outcome and its severity cannot be predicted from genotype data, all patients with PXE should be screened regularly for potential serious and possibly life-threatening complications regardless of their genotype.

An interesting observation was the absence of macroscopic skin lesions in four patients (9, 58, 131 and 171), although skin biopsy revealed typical histological characteristics of PXE. Patient 9 had significant ophthalmological complications, suggesting that this patient was not a carrier. In three of these patients, a complete genotype was determined, confirming the clinical diagnosis and emphasising that skin features, although present in the majority of patients with PXE, are not always mandatory for the diagnosis.

This study is limited by possible selection bias: participants volunteered and were contacted through the support group. Thus, it is skewed towards people who seek support, which might select for people with a more severe phenotype. In addition, more women than men responded, although that gender ratio is reflective of the PXE population in general. Furthermore, the phenotypic data were obtained through self-reports, making their accuracy somewhat questionable, even

though matching with medical records in a subgroup yielded a high correlation. Nonetheless, the results of this study, along with results of all previous genotype–phenotype correlation studies, indicate strongly that a straightforward and clinically usable correlation does not exist, as could be expected from the nature of this disorder. However, because of the significant clinical relevance, further work is needed to determine if there are subcellular phenotypes that correlate with genotypes in PXE.[42] Although mutation detection in PXE has not been shown to have prognostic value, presymptomatic testing in families with history of PXE can provide early diagnosis and may be of value in surveillance for development of disease. In addition, it will probably prove to be of value in genetic counselling as well as in diagnosis of atypical cases or apparent phenocopies.

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Correction

Okada S, Ishikawa N, Shirao K, Kawaguchi H, Tsumura M, Ohno Y, Yasunaga S, Ohtsubo M, Takihara Y, Kobayashi M. The novel *IFNGR1* mutation 774del4 produces a truncated form of interferon-γ receptor 1 and has a dominant-negative effect on interferon-γ signal transduction. *J Med Genet* 2007;**44**:485–91.

The authors apologise for an error in the legend of figure 5. The last sentence should read: ''The cells were treated with CHX (b, d) or untreated (a, c).''

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3.2 Innovative clinical aspects of PXE

Publication 3

Visceral and testicular calcifications as part of the phenotype in pseudoxanthoma elasticum: ultrasound findings in Belgian patients and healthy carriers.

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Br J Radiol 2006;79:221-225

In 2004, a report in the *Journal of Urology* described a fourteen-year old PXE patient in whom coincidentally bilateral testicular microlithiasis was discovered. A possible association of these widespread hyperechogenic foci throughout the testicular parenchyma was suggested, as some previous cases of breast or kidney calcifications in PXE patients were published. Confirmation of such a relationship could have significant clinical implications, as an important number of retrospective studies proposed these lesions as a risk factor for developing testicular cancer.

This study describes the ultrasonographical findings, both abdominal and testicular, in 17 PXE patients and 17 heterozygous carriers, confirming visceral and testicular calcifications to be part of the PXE phenotype in both patients and carriers. Although no abnormalities of abdominal organ function or history of testicular cancer could be beheld, natural history of these lesions is largely unknown. As such, a cautious attitude and regular follow-up should be advised.

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Visceral and testicular calcifications as part of the phenotype in pseudoxanthoma elasticum: ultrasound findings in Belgian patients and healthy carriers

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ABSTRACT. Occasionally calcifications in abdominal organs, breasts and testicles have been reported in pseudoxanthoma elasticum (PXE) patients. In the present study, an ultrasound evaluation was performed of the abdomen and – in male patients – of the testicles in 17 PXE patients and 17 heterozygous carriers. Blood samples were taken to evaluate calcium load, liver and kidney function. Calcifications in liver, kidneys and spleen were detected in 59% of the patients and in 23.5% of healthy carriers. Parameters of kidney and liver function were normal in both groups, suggesting that the calcifications have no direct effect on organ function. Testicular ultrasound revealed parenchymous calcifications in all males investigated. Widespread, small hyperechogenic foci resembling testicular microlithiasis were seen. In some carriers, focal calcifications were identified. The current data suggest that visceral and testicular calcifications are part of the phenotype of PXE patients. Their presence in some of the healthy carriers are suggestive of subclinical manifestations in these relatives. The natural history and long-term effects of the parenchymal calcifications remain to be elucidated. As testicular microlithiasis may be associated with a higher risk for malignancy, regular clinical and ultrasound follow-up seems indicated in these patients.

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Pseudoxanthoma elasticum (PXE – OMIM [Online Mendelian Inheritance in Man]# 264800) is an autosomal recessive connective tissue disorder with multiple systemic manifestations. The phenotype consists of a triad of papular lesions and increased skin laxity in the flexural areas of the body, angioid streaks in the ocular fundus with eventually retinal haemorrhages and loss of central vision, and accelerated atherosclerosis leading to cardiovascular complications [1–5]. The incidence of this rare disease has recently been estimated to be 1:75 000 [6], although this may be an underestimation due to the high variability of the phenotype. Clinical manifestations of the disease are attributed to alterations of elastic fibres within the extracellular matrix of the affected organs. These fibres undergo progressive fragmentation and mineralization, which is the histopathological hallmark of the disease [2]. Nevertheless, other components of the extracellular matrix, such as collagen, fibrillins and proteoglycans have either an abnormal morphology or distribution [7, 8].

The gene responsible for PXE (*ABCC6* - MIM# 603234) is located on chromosome 16p13.1. It encodes an ATP-dependent transporter the substrate of which is as yet unknown. The relationship between this protein and the phenotype also remains to be elucidated [9–11].

It has been shown that healthy carriers of PXE have similar cutaneous abnormalities at the ultrastructural level, suggesting that a mild phenotype may be seen in these individuals [12]. Although a higher incidence of cardiovascular disease has been reported, carriers do not develop other manifestations of PXE such as cutaneous and/or retinal disease [12–14].

Occasionally, PXE patients have been reported in which calcifications in several organs, including kidney, pancreas, spleen and breasts have been observed [15–21]. Additionally, one case report has described the presence of multiple calcifications in the testicles of a 14-year-old PXE-patient [22]. These reports suggest a possible association of organ calcifications and PXE. To our knowledge, no systematic screening of patients nor of healthy carriers has been performed. We present a comprehensive ultrasound study of 17 PXE patients in whom the association between visceral and/or testicular calcifications and PXE was established. Furthermore, 17 heterozygous relatives were screened to detect whether similar lesions could be found.

Patients and methods

Sixteen patients with clinical, molecular and biopsy-proven PXE were examined. Informed consent was

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obtained from all patients and the study was approved by the Ethical Committee of the Faculty of Medicine of the Ghent University Hospital. Our patient population consisted of 5 men and 12 women. Ages ranged from 18 years to 80 years with an average of 54 years.

The group of 17 heterozygous carriers included offspring as well as parents of patients (obligate carriers). Additionally, siblings of patients proven to be heterozygous carriers of an *ABCC6* mutation were included. The carrier group consisted of 11 men and 6 women. Ages ranged from 16 years to 76 years with an average age of 39 years.

All index-patients and carriers were personally examined at the PXE clinic of the Center for Medical Genetics at the Ghent University Hospital. Thorough patient histories were recorded in all individuals with special consideration for signs and symptoms indicating hepatic, renal or splenic dysfunction.

The full clinical protocol used at the PXE clinic of the Center for Medical Genetics at the Ghent University Hospital, including careful dermatological, ophthalmological and cardiovascular examinations and ultrasound of the abdomen and testicles, was applied in both groups. Ultrasound examinations were performed at the Department of Sonography using a HDI 5000 system (Philips, Brussels, Belgium) with a C5-2 and a L12-5 scanhead for the examination of the abdomen and scrotum, respectively. To minimize interobserver variation three ultrasonographers performed the examinations were blinded to patient information. Serum analysis was performed to evaluate calcium load, liver and kidney function in order to exclude other aetiologies of parenchymal calcifications and to assess the possible functional effect of the lesions. Parameters measured in all individuals included serum concentrations of aspartate amino transferase (AST), amino alanine transferase (ALT), alkaline phosphatase (AF), gamma-glutamyl transpeptidase (γGT), creatinine, urea, calcium and phosphorus.

Skin biopsies were taken either in an affected skin area or at the back of the neck when no lesion was macroscopically apparent. Histological confirmation of PXE was obtained with haematoxylin and eosin, van Giesson and Von Kossa stains to detect the typical anomalies of the elastic fibre.

Molecular screening of the *ABCC6* gene was performed using dHPLC (denaturing high performance liquid chromatography) (Transgenomics, Cheshire, UK) and subsequent sequencing of all *ABCC6* exons in those that showed abnormal dHPLC-patterns.

Results

Abdominal ultrasounds

Abdominal ultrasound revealed calcifications scattered throughout the parenchyma of the kidneys (8 patients), liver (4 patients) or spleen (3 patients) in 10/17 (59%) of PXE patients (Figure 1a–d). In those with visceral calcifications, kidneys were most frequently affected (80%). In 3 out of 10 (30%) patients, two or more organs were involved. The number of calcified lesions ranged from a few in the spleen to widely disseminated in the liver parenchyma. Calcifications were seen as hyperechogenic foci with acoustic shadowing, measuring 2–3 mm in diameter. Renal calcifications were localized in the corticomedullary junction, but also within the cortical tissue. Similar lesions could be observed in 4 out of 17 healthy carriers. Two of those had kidney calcifications while the others had lesions in the liver. Other ultrasound findings included hepatic haemangiomas and steatosis.

Serum tests to evaluate kidney and liver function were performed in all patients and carriers examined. No abnormalities of either liver enzymes nor serum creatinine and urea were observed. Calcium levels were always within normal limits. None of the individuals in this study had signs or symptoms indicative of abnormal function of the liver, spleen or kidneys.

Testicular ultrasounds

Ultrasound of the scrotum was performed in four PXE patients. In three multiple widespread, small hyperechogenic foci resembling a ''heaven full of stars'' were identified throughout the parenchyma of both testicles (Figure 2). This appearance matches the criteria of classical testicular microlithiasis as described by Dell'Acqua et al [23]. One patient had only few of these lesions, compatible with limited testicular microlithiasis.

However, no histological confirmation of this diagnosis was obtained since none of the patients had any complaint warranting a biopsy. No testicular tumours were detected during the examination.

In two out of 11 healthy carriers examined, focal calcifications of the testicular capsule or parenchyma were observed. The parenchymatous calcification was a small unilateral focus without acoustic shadowing. These individuals were asymptomatic. Two carriers were found to have a hyperechogenic mediastinum testis, which can be considered a normal variant.

Discussion

PXE is a rare autosomal recessive disease characterized by fragmentation and calcification of the elastic fibres. Clinical manifestations mainly consist of cutaneous, ophthalmological and cardiovascular lesions. Case reports have mentioned the occurrence of calcifications in the visceral organs, breasts and testicles in some individuals [15–21]. In this study, a standardized examination protocol comprising abdominal and testicular ultrasounds was used in 17 PXE patients to observe whether calcified lesions in these organs could be detected.

Due to the autosomal recessive inheritance of PXE, parents and children of probands are obligate carriers of one mutation in the *ABCC6* gene. Previous ultrastructural studies in relatives of PXE patients have revealed cutaneous morphologic alterations similar to those seen in patients, although less severe in nature [12]. Trip et al described a higher risk of coronary artery disease in carriers of the frequent R1141X nonsense mutation [13]. These observations indicate that heterozygous carriers may have mild PXE manifestations, albeit without

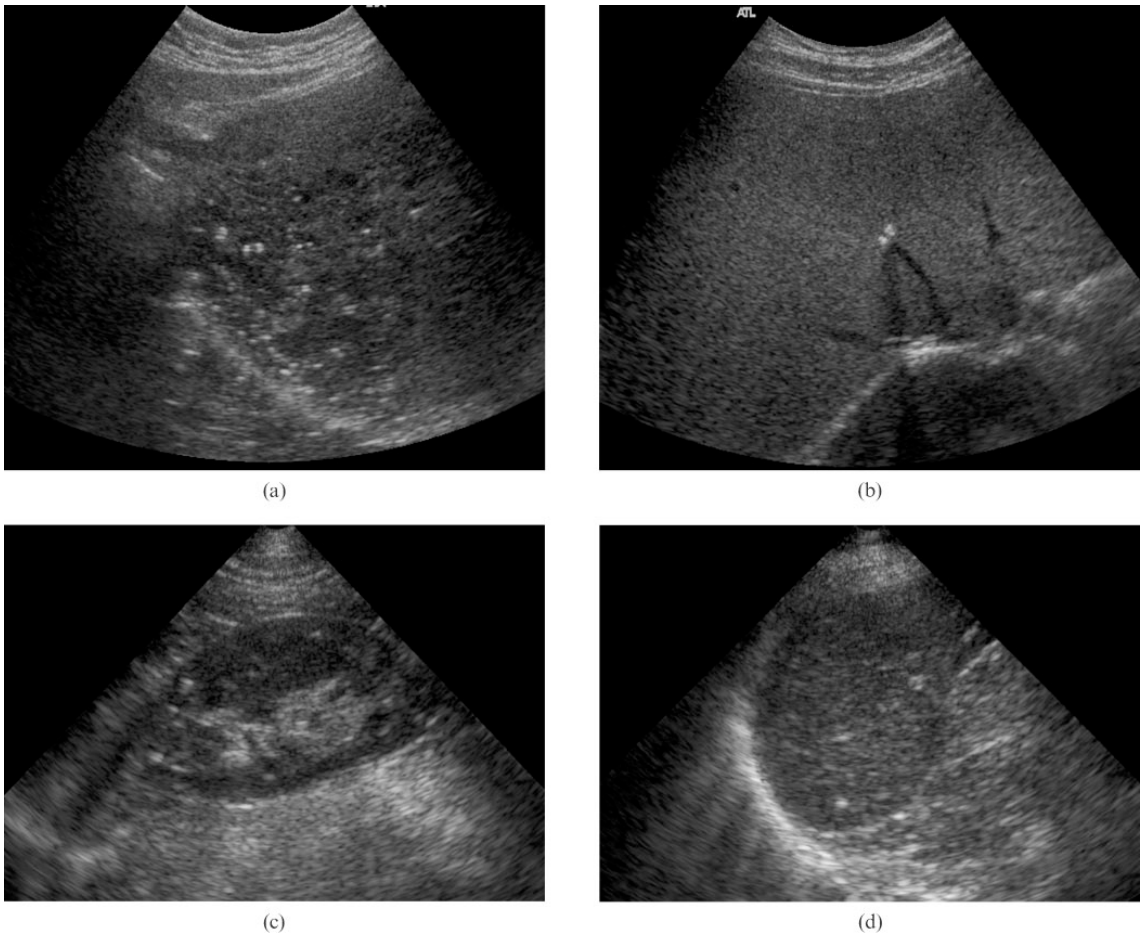

Figure 1. Ultrasound images of calcified foci in several abdominal organs: (a) frontal cross-section through the abdomen with multiple calcifications in the liver of a pseudoxanthoma elasticum (PXE) patient; (b) subcostal transverse cross-section of the liver of a heterozygous carrier in which two calcifications with acoustic shadowing are seen; (c,d) frontal cross-section through the abdomen with view of multiple hyperechogenic foci in (c) the right kidney and (d) spleen of PXE patients.

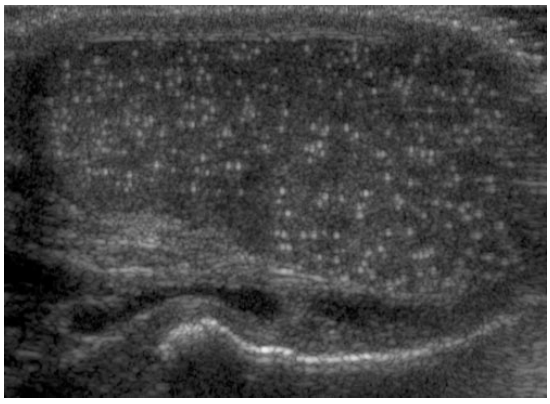

Figure 2. Longitudinal cross-section of the testicle with scattered parenchymatous calcifications in the right testicle of a pseudoxanthoma elasticum (PXE) patient as a typical example of testicular microlithiasis.

obvious cutaneous or ophthalmological symptoms. Therefore, ultrasound evidence of subclinical manifestations was sought in mutation carriers.

Abdominal ultrasound

The data presented suggest that visceral calcifications in the kidneys, liver and spleen are indeed part of the phenotype of PXE patients. Interestingly, similar lesions were found to be present in some of the healthy carriers, although less frequently and to a lesser extent.

All ages were represented in patients and carriers with visceral calcifications, making our findings unlikely to be attributed solely to the age of the individuals. Calcium and phosphorus load were normal in all individuals, excluding other aetiologies of visceral calcifications such as chronic granulomatous diseases (*e.g.* sarcoidosis), renal failure, hyper(para)thyroidism, pheochromocytoma, adrenal insufficiency or malignancy.

As serum tests for liver and kidney function revealed no abnormalities and none of the individuals examined suffered from any disturbances of renal, hepatic or splenic function, the calcified foci probably do not interfere with liver, kidney or splenic function. However, their natural history and long-term effects remain to be elucidated.

Therefore an abdominal ultrasound at the time of diagnosis may be indicated. Furthermore, regular re-evaluation with serum tests and ultrasound are advisable.

It has been previously reported that both abdominal plain radiographs and CT are unable to visualize these lesions [18]. In the only patient with renal foci in whom abdominal radiographs were performed in this study, no calcifications were visible. We did not perform CT imaging in our population and can therefore not rule out that, due to technical improvements and new developments, these lesions can now be visualized. However, since ultrasound proved to give sufficient data and comparing the costs and radiation load of both examinations, we feel that at present CT is not an added value in the work-up of a PXE patient in a clinical setting. In a research setting, however, it would be interesting to find out if these lesions are indeed visible with modern CT techniques and to evaluate their extent and characteristics in comparison with ultrasound findings. Thus, in view of all known aspects, ultrasound should be considered the investigation of first choice for detection of these calcifications on a routine basis.

Testicular ultrasounds

Testicular parenchymal calcifications were identified in all male patients so far examined. These lesions, described as bilateral, small, hyperechogenic foci, meet the ultrasound criteria of testicular microlithiasis (TM). The TM pattern is defined as usually bilateral hyperechogenic multiple small foci without acoustic shadow and with complete or partial extension to the parenchyma. Cases in which five or more foci can be demonstrated are defined as classical TM [24–27]. Cases that do not meet this criterion are designated as limited TM. The imaging diagnosis can be confirmed by showing intratubular microliths on biopsy [24–27]. Since none of our patients had either complaints or fertility problems testicular biopsies were considered unethical. TM is of special interest due to its reported association with testicular malignancy [29–36]. Nevertheless, it remains unclear whether primary testicular tumours actually occur more frequently in patients with pre-existing TM. Large prospective studies are needed to further clarify this issue. Until further data are available, it seems cautious to consider patients with a TM-like ultrasound image as having a potentially increased risk of developing a testicular malignancy and to offer regular ultrasound screening [28–34, 36].

The findings in healthy carriers were different from those in patients in their extent and/or location within the testicle. Multiple hyperechogenic foci confined to the capsule or the mediastinum testis were detected, the latter probably being a normal variant. Although anatomically this could also be compatible with calcifications in the rete testis [37], we cannot be sure of this without a biopsy which is unjustifiable in these patients.

In another carrier, we observed one parenchymatous calcification which could be considered as limited TM. The remaining parenchyma, however, was completely normal and we cannot exclude that these findings are fortuitous. Since they have, to our knowledge, not previously been described in PXE, further study on a larger group of carriers would be of interest.

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Publication 4

Pseudoxanthoma elasticum with generalized retinal dysfunction, a common finding?

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In this study, four unrelated PXE patients presenting with unexplained vision loss – i.c. decreased visual acuity in the absence of retinal haemorrhages – were investigated thoroughly via electrophysiological examinations to assess retinal and macular function.

Three distinct types of retinal dysfunction could be delineated, suggesting that such malfunction is not uncommon in PXE patients, particularly when no obvious anatomical corruption can be detected. In addition, molecular analysis in these patients revealed a novel *ABCC6* nonsense mutation.

Pseudoxanthoma Elasticum with Generalized Retinal Dysfunction, a Common Finding?

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Purpose. Pseudoxanthoma elasticum (PXE; [MIM 264800]) is an autosomal recessive systemic disorder characterized by progressive degeneration and calcification of elastic fibers in connective tissue. The phenotype is variable, with cutaneous, vascular, and ophthalmic abnormalities. The disorder is a consequence of mutations in the *ABCC6* gene. Visual impairment is mainly due to neovascular complications, and retinal function is usually assumed to be normal. The purpose of this study was the objective assessment of macular and generalized retinal function in unrelated patients with clinical and/or genetic features of PXE.

Methods. Four unrelated patients carrying a clinical diagnosis of PXE presented with unexplained visual loss. After ophthalmic examination, retinal and macular function was assessed by full-field electroretinogram (ERG) and pattern ERG, respectively, according to ISCEV (International Society for Clinical Electrophysiology of Vision) recommendations. Molecular analysis of the *ABCC6* gene was performed in three patients by dHPLC (denaturing high-performance liquid chromatography) and direct sequencing.

Results. Full-field ERG revealed significant reduction of cone and rod responses in all four patients. Funduscopic appearances varied. Three patients were found to carry *ABCC6* mutations. In case 1, a novel nonsense mutation (p.L1474X) was detected in exon 31 paired with a splice-site mutation. Mutation analyses in cases 3 and 4 revealed previously reported *ABCC6* mutations.

Conclusions. These findings suggest that retinal dysfunction in PXE may not be uncommon. The mechanism underlying retinal dysfunction is unknown but may result from metabolic disturbance leading to retinal toxicity with a possible role of modifying genetic or environmental factors rather than specific *ABCC6* mutations. (*Invest Ophthalmol Vis Sci.* 2007;48: 4250–4256) DOI:10.1167/iovs.05-1604

Pseudoxanthoma elasticum (PXE, MIM [Mendelian Inheritance in Man] 264800) is a systemic disorder characterized by progressive degeneration and calcification of elastic fibers. The phenotype of the disease is highly variable, partially depending on the age of the patient, and includes mainly skin, ocular, and cardiovascular abnormalities (see Ref. 1 for review). The prevalence of the disease is estimated between 1 in 70,000 and 1 in 100,000.[2,3] In the past, both autosomal recessive and dominant transmission have been described. However, recent evidence suggests that most, if not all, familial cases are recessive forms. Previously described dominant pedigrees can be explained by the presence of pseudodominance due to a high carrier rate or consanguinity.[3,4] Several groups have identified *ABCC6* as the defective gene,[5–8] which causes a loss of function resulting in the PXE phenotype. It is a member of the ATP-binding cassette transmembrane transporter family, also assigned to the subfamily of multidrug resistant proteins (MRPs).[9] It is highly expressed in human and mouse liver, to a lesser degree in kidneys,[10–12] but at only very low levels in the tissues that are clinically most affected.[5,10] The precise function of ABCC6 remains unclear, although its location at the basolateral side of the cellular membrane suggests a role in transporting substances involved in connective tissue homeostasis, normally extruded from liver and kidney. Hence, PXE could be considered a metabolic disease.[13,14]

The cutaneous phenotype typically consists of discolored (yellowish) papules and plaques on the neck and Moroccan leatherlike skin lesions with redundant folds in flexural areas. Cardiovascular complications that may be associated with the disease are mainly due to accelerated atherosclerosis (e.g., peripheral and coronary artery disease, stroke).[1]

The ocular phenotype is variable. A "peau d'orange" fundus appearance can be present in childhood as the earliest ophthalmic sign, but it tends to become less distinct with age. This unusual mottled feature corresponds with yellowish lesions of the retinal pigment epithelium (RPE), typically in the midperiphery and particularly on the temporal side. Angioid streaks radiating from the optic disc are commonly reported, and other signs may include optic nerve head drusen and reticular pigmentary dystrophy of the macula. Crystalline bodies may be present in the midperiphery and juxtapapillary area with variable degrees of underlying RPE atrophy. These lesions are referred to as "comets" and may be associated with streaks of RPE thinning in a cometlike tail pattern extending toward the posterior pole.[15,16] These focal abnormalities may be the only lesions typical of PXE, according to Gass.[15]

Visual impairment in patients with PXE can occur because of macular atrophy, choroidal rupture, or choroidal neovascularization, with or without choroidal hemorrhage from angioid streaks, and theoretically from complications of optic nerve head drusen. Visual function is poorly documented in the literature: François and De Rouck[17] described mild amplitude

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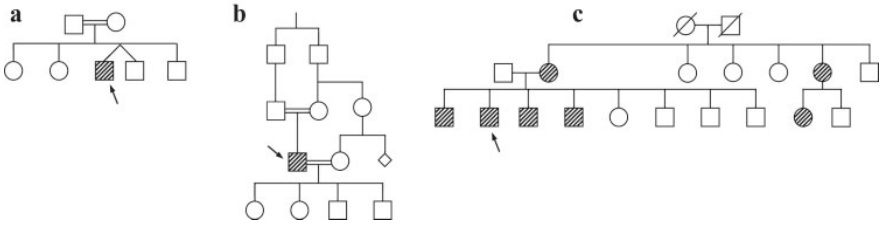

FIGURE 1. Family pedigrees. (**a**) Case 1: there was no family history of ocular disorder or PXE. Parents were first cousins. The patient had an unaffected dizygotic twin. (**b**) Case 2: no family history of ocular disorder or PXE. Parents were first cousins and the patient married one of his first cousins. (**c**) Case 4: the patient had three affected siblings with variable PXE manifestations as well as an affected mother, maternal aunt, and a cousin, which raised the suspicion of pseudodominant PXE.

reduction in electroretinograms (ERGs) in 46% of cases, mostly in eyes with advanced disciform macular degeneration suggesting that retinal dysfunction is not an unusual abnormality in PXE. Holz et al.[18] failed to detect any functional changes associated with the peau d'orange retinal appearance.

In this study, four patients with PXE are reported who had generalized retinal dysfunction involving both cones and rods.

METHODS

Electrophysiology was performed with gold foil recording electrodes according to ISCEV (International Society for Clinical Electrophysiology of Vision) standards.[19,20] Molecular analysis was performed in three patients. The *ABCC6* gene was amplified by using polymerase chain reaction primers previously described by Wang et al.[21] and Le Saux et al.[22] For the detection of the common exon 23 to 29 deletion, a PCR-based method with primers described by Le Saux et al.[22] was performed on all samples. In this assay, PCR amplification will be observed only in the presence of a multiexon deletion. The coding region and intron–exon boundaries of the whole *ABCC6* gene were analyzed with dHPLC (denaturing high performance liquid chromatography; Wave System; Transgenomics, Inc., Omaha, NB) and direct sequencing (model 3100 sequencer with PRISM BigDye Terminator Cycle Sequencing Kit; Applied Biosystems, Inc., Foster City, CA).

The protocols used to performed this study adhered to the tenets of the Declaration of Helsinki and were approved by the local Ethics Committee.

RESULTS

Case 1

A 16-year-old Turkish boy had a history of night blindness since the age of 2. Although fit and well at presentation, he had been treated for non-Hodgkin malignant lymphoma at the age of 2 years with chemotherapy, including systemic treatment with endoxan, oncovin, ara-C, methotrexate, and 6-mercaptopurin and intrathecal methotrexate, prednisolone, and alexan. He reported poor color discrimination, worsening of visual acuity, and progressive visual field constriction over a 3-year period. There was no family history of visual problems or systemic diseases. His dizygotic twin brother was asymptomatic with normal vision, and his parents were first cousins (Fig. 1a). At the time of his first visit, his vision was 6/12 in both eyes with an optical correction of $-4(-1.75)20°$ in the right eye and $-3.75(-2.25)165°$ in the left. He had been wearing spectacles since the age of 5. Static perimetry (Fig. 2) showed diffuse loss of sensitivity worse in the central field. On slit lamp examination, his anterior segments were unremarkable, and, in particular, there was no evidence of crystalline deposits in the corneal limbus. Fundus examination (Fig. 3a) revealed bilateral angioid streaks, a peau d'orange aspect on the temporal side of the macula of both eyes and some macular RPE atrophy. Intraretinal crystalline bodies were disseminated over the posterior pole and midperiphery and associated with underlying RPE atrophy. Some of the larger crystalline lesions were associated with a punched-out appearance. Fundus autofluorescence examination (Fig. 3b) showed areas of low density consistent with RPE atrophy associated with the crystals, angioid streaks, and fovea. The punched-out lesions had a distinct autofluorescent appearance with a high-density center surrounded by a hypoautofluorescent ring. Angioid streaks and the crystalline lesions showed areas of window defect on fluorescein angiography congruent with the underlying RPE atrophy (Fig. 3c). On indocyanine green angiography, punched-out lesions correspond to hypofluorescent areas (Fig. 3d). Examination with optical coherence tomography (Fig. 3e) revealed the presence of crystalline bodies at the level of the inner retinal layers. Color contrast sensitivity was assessed along the protan, deutan, and tritan axes. All thresholds were grossly elevated (data not shown). The patient underwent electrophysiology (Fig. 4a). Pattern ERG was markedly reduced in the right eye and undetectable in the left. The rod-specific ERG was markedly subnormal in both eyes. The maximum responses showed reduced amplitude for both a- and b-waves. The 30-Hz flicker and single flash cone ERGs showed profound delay and profound reduction in amplitude. The findings are those of severe generalized retinal dysfunction involving the cone more than the rod systems, with pattern ERG evidence of macular involvement, worse on the left than the right. Other tests results, including full blood count, ionogram, creatinine level and hemoglobin electrophoresis, were normal. No sickle cell trait was detectable. A dermatologic examination showed no evidence of the skin lesions in the flexural areas, and the patient declined a skin biopsy. Visual acuity dropped to 6/24 bilaterally 32 months after presentation but the fundus appearance was unchanged.

Molecular analysis of the *ABCC6* gene revealed two base-changes: a 3507(–3)C→T transition at the splice acceptor site of intron 24 and a p.L1474X nonsense mutation (c.4420 A→T, exon 31; Figs. 5a, 5b).

Case 2

This 45-year-old Moroccan man presented with a 2-year history of gradual visual loss in both eyes and photophobia. He also reported difficulties seeing in the dark and especially adjusting from light to dark. Six years earlier, he had been given a diagnosis of PXE based on typical skin lesions and fundus abnormalities. There was no family history of visual problems or systemic disorders. His parents were first cousins (Fig. 1b). His visual acuity at the time of the referral was hand movements with $+1(-1.50)90°$ on the right and 6/18 to 2 with $+0.50(-1.25)90°$ on the left. Visual fields to confrontation

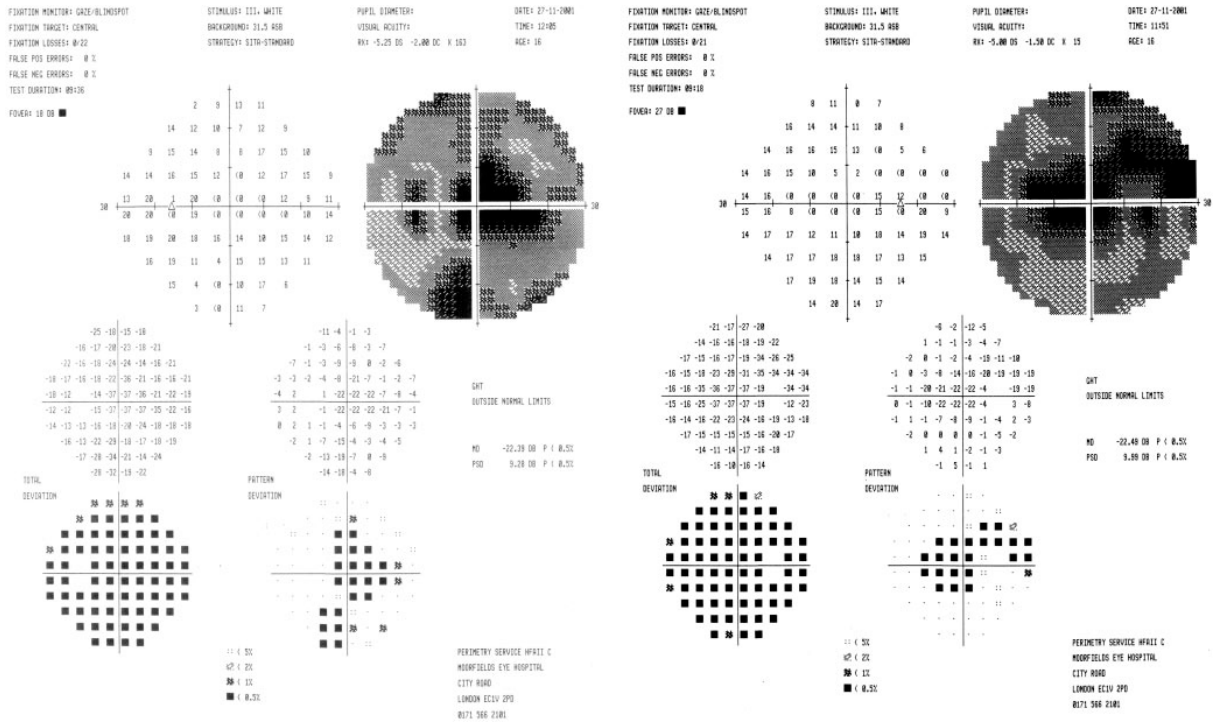

FIGURE 2. Case 1: static visual field tests: note the diffuse loss of sensitivity worse in the central field.

showed residual perception in the superior and temporal field of both eyes. A fundus examination (Fig. 3f) showed healthy optic discs, narrow vessels, angioid streaks, and bilateral pigmented fibrovascular scars in the macula, probable sequelae of bilateral choroidal neovascularization. A peripheral examination showed atrophy of the RPE outside the arcade, with pigment migration at the level of the retina.

Electrophysiology was performed (Fig. 4). No pattern ERG was detectable. A full-field ERG showed no rod-specific response, severely subnormal maximal rod– cone responses and markedly delayed and reduced 30-Hz flicker ERG. The findings were consistent with severe generalized retinal dysfunction affecting both rod and cone systems, with severe bilateral macular involvement. These results suggest advanced photoreceptor dystrophy and cannot be explained on the basis of fibroglial scars present on fundus examination. He was reviewed in the clinic 6 months later and showed a decline in his vision-to-hand movement in the right eye and 6/36 in the left. Two years after presentation, his vision was hand motion in the right eye and 6/60 with +1.50(−1.50)85° in the left. The fundus appearance was unchanged. The patient declined further testing and was unwilling to provide a blood sample for genotype analysis.

Case 3

This 37-year-old white woman with a diagnosis of PXE was referred in May 2003 for follow-up of her retinal status. She reported night blindness and peripheral visual field constriction. She had received a diagnosis of PXE in her early 20s, based on characteristic fundus abnormalities and typical skin lesions. There was no family history of ocular disease or systemic disorder and there was no parental consanguinity. Her visual acuity was 6/9 in the right eye with −1.50 D and 6/24 with −1 D in the left eye. Visual fields showed concentric constriction in her right eye and diffuse loss of sensitivity in the left eye. Fundus examination (Fig. 3g) revealed multiple angioid streaks, a temporal appearance of peau d'orange, punched-out lesions in the midperiphery, and RPE atrophy in the periphery with pigment migration into the neural retina. There was no evidence of choroidal neovascularization. One of the angioid streaks passed through the foveola in the left eye, with additional RPE atrophy explaining the poor left visual acuity. Pattern ERG and full-field ERG abnormalities were severe bilaterally, in keeping with generalized retinal dysfunction affecting rod more than cone photoreceptors with evidence of severe bilateral macular involvement (Fig. 4). Her vision remained stable, and her fundus appearance was unchanged at the 1-year follow-up.

On mutation analysis, this patient was homozygous for the p.R1141X nonsense mutation (c.3421C→T) in exon 24 (Fig. 5c).

Case 4

This white man was first seen at the age of 26. He carried a diagnosis of PXE based on fundus changes (angioid streaks and peau d'orange), typical skin lesions, and a history of digestive complications with gastrointestinal hemorrhage. The diagnosis was confirmed by skin biopsy, which showed degeneration and calcification of the elastic fibers.

He had visual field loss that initially had been attributed to bilateral complicated optic nerve head drusen. He also had three affected siblings with variable manifestations of PXE as well as an affected mother, maternal aunt, and a cousin. There was a possible low degree of consanguinity in the family, which raised the suspicion of pseudodominant PXE (Fig. 1c).

His vision at the time of the first referral was 6/18 in the right eye and 6/9 in the left. Over the course of follow-up, his visual acuity gradually deteriorated consequent to choroidal

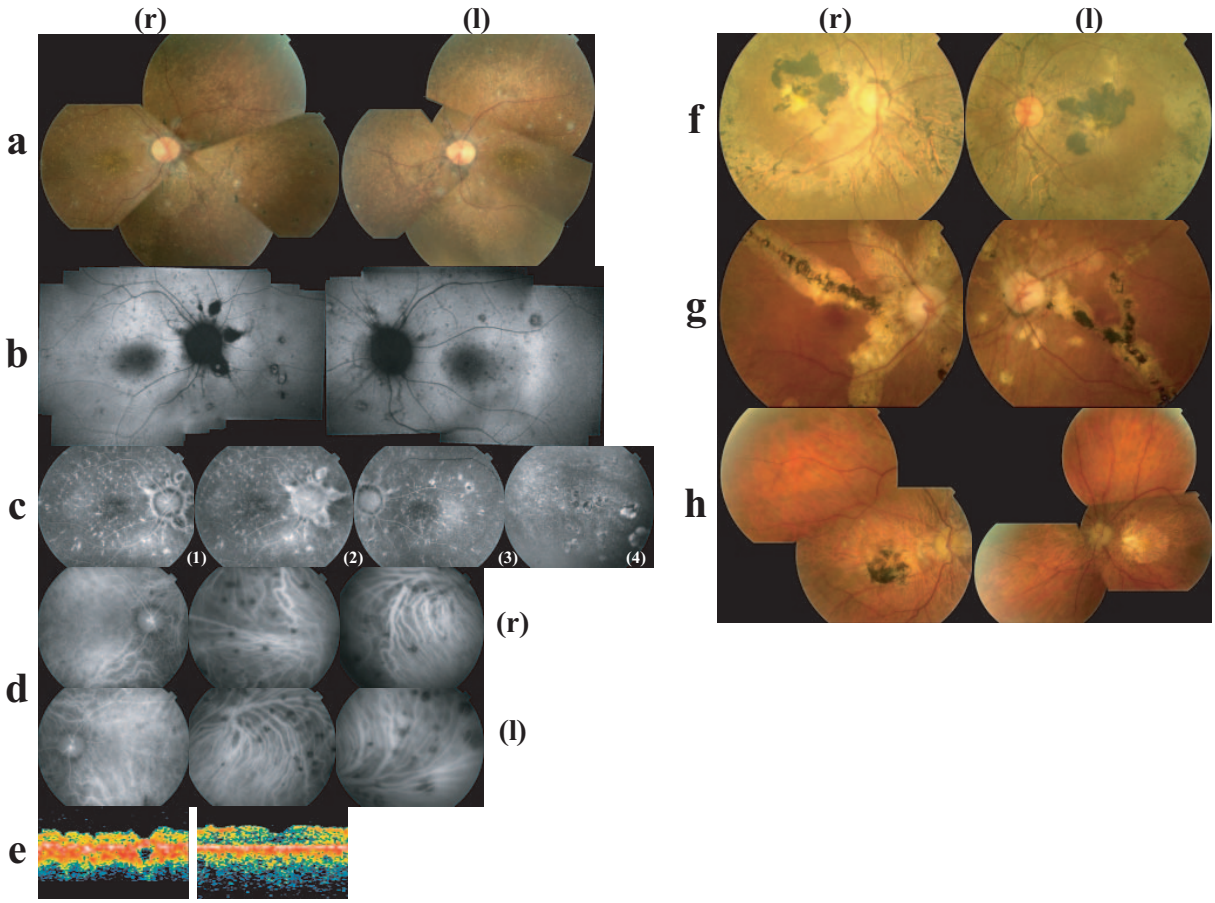

FIGURE 3. (**a**) Case 1: fundus color photographs from the right (r) and left eye (l). Note the presence of bilateral angioid streaks, peau d'orange in the temporal sector of both eyes, some amount of macular retinal pigment epithelium atrophy, atypical intraretinal crystalloid deposits disseminated over the posterior pole and midperiphery, cometlike tail aspect with underlying retinal pigment epithelium atrophy. Some of the larger crystalline lesions have a punched-out appearance. (**b**) Fundus autofluorescence. Note the areas of low-density consistent with RPE atrophy associated with the crystals, angioid streaks, and fovea. The punched-out lesions have a distinct autofluorescence appearance with a high-density center surrounded by a hypoautofluorescent ring. (**c**) Fluorescein angiography from the right eye (54s (1) and 5m36s (2)) and from the left eye (16s (3) and 3m40s (4)). Note the hyperfluorescent area associated with the angioid streaks, the crystals and the punched-out lesions by window defect reflecting the underlying RPE atrophy. (**d**) Indocyanine green angiography: hypofluorescent spots corresponding to the punched-out lesions. (**e**) Optical coherence tomography: note the presence of crystals in the inner retinal layers. (**f**) Case 2: healthy optic discs, narrow blood vessels, angioid streaks, and bilateral pigmented fibrovascular scars in the macula, probable sequelae of bilateral choroidal neovascularization. A peripheral examination showed atrophy of the retinal pigment epithelium outside the arcade with pigment migration at the level of the retina, a sign of photoreceptor loss. (**g**) Case 3: multiple angioid streaks including one that passes through the foveola in the left eye and additional atrophy of the retinal pigment epithelium, explaining the low vision in the left eye; no evidence of choroidal neovascularization; a temporal appearance of peau d'orange; punched-out lesions in the midperiphery; RPE atrophy in the periphery with pigment migration and bone spicules at the retinal level. (**h**) Case 4: waxy appearance of both optic nerve heads and drusen, angioid streaks, a fibroglial scar on the right macula, and an area of geographic atrophy on the left.

neovascularization in the right eye, which resulted in a disciform scar, and atrophic changes in the left eye, in addition to the optic nerve drusen. By the age of 55, his vision was hand movements in the right eye and 1/60 in the left eye. Fundus examination (Fig. 3h) showed a waxy appearance of both optic nerves with nerve head drusen, angioid streaks, a fibroglial scar on the right macula and an area of geographic atrophy on the left. The severity of visual impairment was unusual for PXE and electrodiagnostic tests were performed to distinguish between optic nerve and retinal dysfunction (Fig. 4). No pattern ERG was detectable, indicating severe macular dysfunction bilaterally. Rod specific ERG was subnormal. Maximum ERGs, 30-Hz flicker, and transient photopic ERGs were mildly subnormal bilaterally. These findings were consistent with severe bilateral macular dysfunction with evidence of mild generalized retinal involvement affecting the rod more than the cone system.

The patient was found to be compound heterozygous for the known p.R1114P missense mutation (c.3341G→C) in exon 24 and a multiexon deletion of exons 23 through 29 (Figs. 5d, 5e).

DISCUSSION

The present study characterizes the molecular and clinical features of a heterogeneous group of four patients with PXE who underwent comprehensive assessment of generalized retinal function. Full-field ERG revealed three functional pheno-

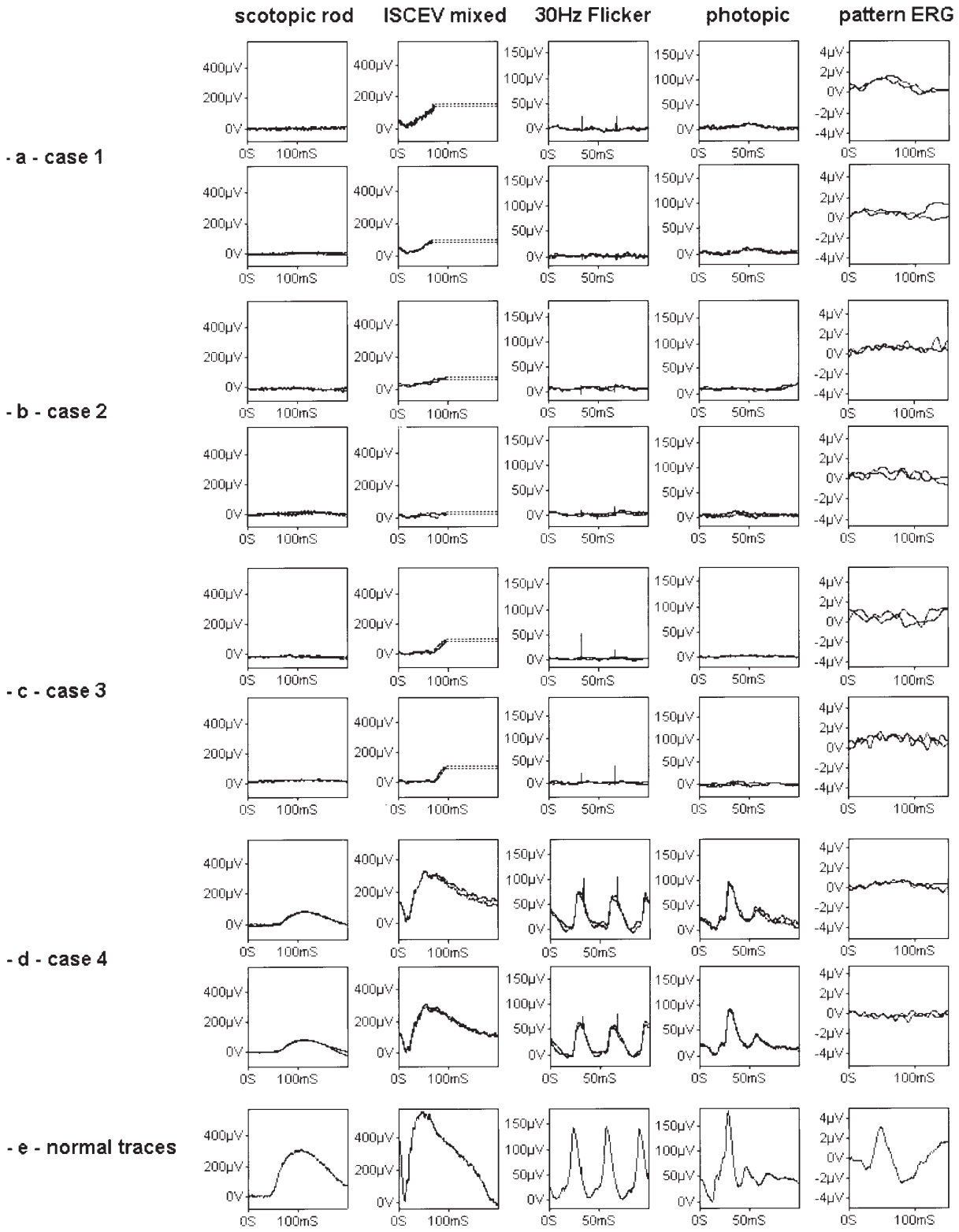

Figure 4. Electrophysiology (**a**) Case 1: scotopic rod-specific ERGs are markedly subnormal from both eyes. The maximum responses show reduced amplitude for both a- and b-waves. Both 30-Hz flicker and single flash cone ERGs show profound delay and profound reduction in amplitude. Pattern ERG is markedly reduced in the right eye (RE) and undetectable in the left (LE). The findings are those of severe generalized

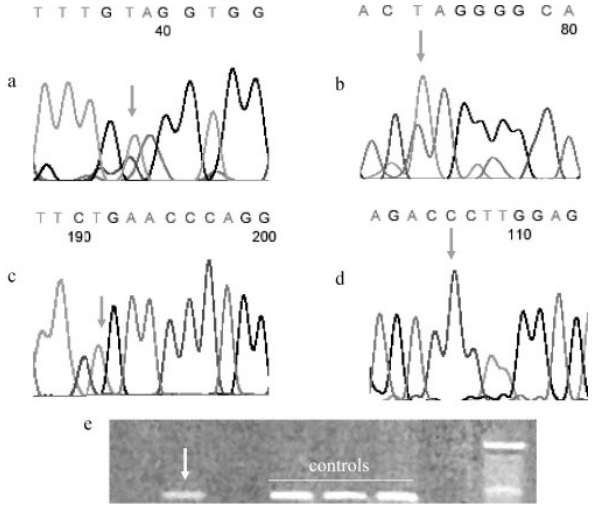

FIGURE 5. Electropherograms of the sense strand of genomic DNA from the patients investigated (**a**–**d**) and an agarose gel electrophoresis picture of patient 4 (**e**). A heterozygous splice-site mutation (**a**, 3507(−3)C→T) and heterozygous nonsense mutation (**b**, c.4420A→T) in case 1, a homozygous nonsense mutation **c**, c.3421C→T) in case 3, and a heterozygous missense mutation (**d**, c.3341G→C) in case 4. Note that only the undeleted allele amplified. The presence of a multiexon deletion (**e**, del23-29) with specific primers with positive controls is shown in case 4. *Arrows*: position of the mutation.

types; cone–rod dystrophy (case 1), rod–cone dystrophy (cases 3 and 4), and severe photoreceptor dystrophy involving rods and cones equally (case 2). Uniquely in PXE, pattern ERGs were recorded, allowing objective assessment of macular function.[23]

Funduscopic appearances also varied. Diffuse changes extending beyond the arcades were seen (cases 1 and 2) with numerous intraretinal crystals in case 1. One possibility is that the crystalline retinopathy is due to the chemotherapeutic agents used during his childhood illness, although there is no precedent for this with any of the agents listed. Alternatively, this appearance has been reported in the retinas of persons affected with PXE,[1,15] whereas no association between the chemotherapeutic drugs taken by the subject and crystals can be found in the literature. In case 1, a novel nonsense mutation (p.L1474X) was detected in exon 31. A further base change was detected, 3507(−3)C→T, which is predicted to affect splicing and has been detected previously in PXE patients (Olivier Vanakker, personal communication, 2005). Mutation analysis in cases 3 and 4 revealed known mutations in exon 24 and a multiexon deletion which is frequently observed in PXE.

There have been few reports in the literature describing retinal function in PXE. François and De Rouck[17] described 15 patients. Slightly subnormal dark adaptation was present in 18 eyes. Nonstandard ERG recordings suggested mild retinal dysfunction in 14 eyes that involved only the cone system (2 cases), only the rod system (4 cases), or both rod and cone systems (8 cases). Most of these patients had advanced disciform degeneration, although normal ERGs were also recorded in cases of terminal macular degeneration. These findings suggest that moderate retinal dysfunction, similar to that in case 4 (Fig. 4d) may commonly be present and could explain the night vision difficulties reported frequently by PXE patients in our experience.

Yoshida et al.[24] described an association between PXE and severe retinal dysfunction in a single Japanese pedigree.[24] The proband was homozygous for a novel mutation in *ABCC6* (nonsense mutation in exon 5), and his two affected daughters were heterozygous for this mutation with clinical signs of PXE and ERG evidence of rod–cone dystrophy (RP). The authors suggested either a coincidental association or the possibility of a combination of *ABCC6* alleles, which can give rise either to PXE or PXE and RP. *ABCC6* may also behave like a modifier gene contributing to the pathogenesis of RP.[24]

A coincidental association between PXE and retinal dystrophy in the current series could occur, with co-inheritance of the *ABCC6* mutations with those in an as yet unknown gene with a role in retinal function. This scenario would be more likely in cases 1 and 2, for whom parental consanguinity theoretically increases the likelihood of an association of two separate and independent recessive disorders. The generalized retinal dysfunction seen in these four patients may have been due to the effect of specific alleles of the *ABCC6* gene. However, the genetic analysis of three patients suggests not. Five of the six mutant alleles are known to contribute to PXE in other studies and are considered to represent null alleles. The one novel mutation detected in this study, p.L1474X, would cause a premature-truncating codon and would be likely to succumb to nonsense-mediated decay and hence also to be null. It is more likely therefore that the determination of whether PXE will be accompanied by generalized retinal dysfunction will depend on modifying factors, either environmental or genetic, that affect the way retinal tissue tolerates the metabolic abnormalities of the disorder.

Why retinal tissue should be affected at all by *ABCC6* disruption is not clear. Only low levels of *ABCC6* mRNA have been detected in frequently affected tissues,[5,10,12] although conflicting results have been reported in the literature regarding immunohistochemical analysis of the ABCC6 protein.[5,9,12,13] Conversely, high levels of ABCC6 mRNA and protein were found in liver, which is unaffected in PXE.[5,10–13] Gorgels et al.[13] observed a decrease in HDL cholesterol levels in *Abcc6*$^{-/-}$ mice and suggested that alteration of plasma lipid composition may be implicated in the systemic changes observed in PXE. Retinal toxicity could be secondary to changes in Bruch's membrane precluding proper nutrient supply to photoreceptors leading to their secondary dysfunction and degeneration. The further investigation of *Abcc6*$^{-/-}$ mice might give insights into the mechanisms that lead to the pathologic changes observed in PXE.[13]

retinal cone–rod system dysfunction with macular dysfunction worse on the left than on the right. (**b**) Case 2: full-field ERGs show an undetectable rod-specific response, severely subnormal maximum responses and markedly delayed and reduced-flicker ERG. No definite pattern ERG is detectable. The findings are those of severe generalized retinal dysfunction, affecting both rod and cone photoreceptors with severe bilateral macular involvement. (**c**) Case 3: the scotopic rod-specific ERG was severely reduced bilaterally. Maximum responses are markedly subnormal. The 30-Hz flicker is delayed and subnormal in amplitude as is the photopic single-flash ERG. Pattern ERG is bilaterally undetectable. The findings are consistent with a moderately severe photoreceptor dysfunction affecting the rods more than the cones with severe bilateral macular involvement. (**d**) Case 4: scotopic rod-specific ERGs are subnormal. The 30-Hz flicker and transient photopic ERGs were mildly subnormal bilaterally. No pattern ERG was detectable. The findings are consistent with generalized rod dysfunction with mild cone system involvement and with bilateral macular dysfunction. (**e**) Example of normal responses. Maximum ERGs were recorded to a stimulus 0.6 log units stronger than the ISCEV standard flash, as a better demonstration of the ERG a-wave.[19]

When considering the influence of *ABCC6* mutations on retinal function, electrophysiological assessment provides important insight into the underlying causes of visual failure and most accurately describes the functional phenotype. It should probably be considered more often, although it would normally be indicated if any discrepancy exists between the patient's visual complaints, visual acuity, and clinical examination.

In conclusion, the present study highlights an underreported but disabling association of PXE—generalized retinal dysfunction—and describes the detailed phenotype of these patients. It appears that this specific complication of PXE is not due to the inheritance of specific alleles, which suggests the action of modifiers. It is possible that the generalized retinal dysfunction in PXE is more common than is recognized.

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Publication 5

Added value of infrared, red-free and autofluorescence fundus imaging in pseudoxanthoma elasticum.

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Successful medical care for PXE patients is strongly depending on the age of diagnosis; early disease recognition implies a better prognosis. This is particularly true for the ophthalmological complications, for which several therapeutic options nowadays exist. From this perspective, novel techniques became available – often for other, more common disorders – which can be of potential interest for PXE families.

In this study three innovative fundus visualization methods – infrared, redfree and autofluorescence imaging – are evaluated for their capacity to depict the PXE retinopathy in 22 PXE patients and 25 obligate carriers. These techniques emerged as standard imaging instruments in age-related macular degeneration and several other retinal dystrophies. Particularly the wet form of ARMD, characterized by neovessel formation, reveals great similarity with the PXE retinopathy and triggered the hypothesis of better visualizing early onset ocular disease in PXE. Indeed, these techniques were shown to be more sensitive than white light funduscopy in visualizing early retinal changes in PXE, as well as appreciating their extent. It is concluded that they should be considered part of the standard work-up of PXE families.

Added Value of Infrared, Red-free and Autofluorescence Fundus Imaging in Pseudoxanthoma Elasticum

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Abstract

Purpose: Pseudoxanthoma Elasticum (PXE) is an autosomal recessive disorder caused by mutations in the *ABCC6* gene, and primarily affects the oculocutaneous and cardiovascular systems. However, the phenotype, including the ophthalmological manifestations, varies in severity. The present study aims to evaluate the added value of novel fundoscopic imaging techniques, such as infrared, red-free and autofluorescence imaging in PXE.

Methods: In 22 molecularly proven PXE patients and 25 obligate carriers with one *ABCC6* mutation, PXE retinopathy was evaluated using funduscopy, white light, red-free, infrared and autofluorescence imaging.

Results: At least one characteristic of PXE retinopathy was evident on fundoscopy of all eyes. Angioid streaks could be subdivided in those with (brick red) or without (feathered) adjacent RPE alterations. Infrared imaging showed the brick red coloured streaks as well-demarcated dark fissures, even when these passed unnoticed on funduscopy. Feathered types were detected as triangular areas of hypo-autofluorescence. The peau d'orange was much better visible and much more widespread on infrared imaging, with extension from the posterior pole towards the whole midperiphery. Comets and comet tails, were best seen with red-free imaging.

Conclusions: Infrared, red-free and autofluorescence imaging are more sensitive than white light funduscopy and imaging in visualizing early retinal signs of PXE. In addition, this specialised imaging allows better appreciation of the extent of lesions. Hence, such imaging increases the chances of making a correct diagnosis early, and aids in the accurate evaluation of evolution of disease in the ophthalmic follow-up of PXE patients.

Introduction

Pseudoxanthoma elasticum (PXE; OMIM# 264800) is an autosomal recessive multisystem disorder, mainly affecting the oculocutaneous and cardiovascular systems[1,2,3]. It is caused by mutations in the *ABCC6* gene (chrom. 16p13.1; OMIM# 603234), encoding a transmembrane transporter of which the substrate remains as yet unknown[4,5]. The histology of PXE is characterized by ectopic mineralization and fragmentation of elastic fibres in the reticular dermis, Bruch membrane (BM) and the elastic laminae of blood vessels[1,2].

Skin symptoms manifest first in childhood as yellowish discoloured papules or increased skin laxity in flexural areas, although there is significant variability in clinical severity[1,2,3]. Cardiovascular complications are those of accelerated atherosclerosis with occlusive vascular disease[1,2,3]. The PXE retinopathy is characterized by a mottled aspect of the retinal midperiphery (called peau d'orange) most prominent temporal to the macula, and ruptures in BM known as angioid streaks. The latter often, if not invariably, lead to subretinal neovascularisation with a risk for spontaneous and/or post-traumatic haemorrhage with subsequent loss of visual acuity[1,2,3]. Additionally, comets and comet tails, crystalline bodies in the retinal midperiphery and juxta-papillary area with a variable degree of retinal pigment epithelium (RPE) atrophy have been reported. Although not known to impair visual function, they have been described as the only lesions pathognomonic for PXE[6]. The clinical heterogeneity of PXE is also reflected in the retinal phenotype: although invariably present, the retinopathy remains rather limited and uncomplicated in young patients, making an early diagnosis more difficult. Additionally, very similar ophthalmological characteristics have been observed in PXE phenocopies, such as those associated with either beta-thalassaemia or the PXE-like syndrome (OMIM# 610842) [8,9]. However, ocular signs and symptoms are less severe and uncomplicated in the latter[9].

Dilated fundus photography using white light, whether or not combined with fluorescein angiography, has proven to be a useful technique to evaluate the retinal phenotype. More recently, novel non-invasive imaging techniques, such as infrared imaging (IRI) and autofluorescence imaging (AFI), have emerged[10-15]. The former allows evaluation of the integrity and health of the RPE and the underlying choroidal structures. Infrared light penetrates more easily through the optical media and has a reduced absorption and increased reflection both by melanin and haemoglobins when compared to light of wavelengths shorter than 585 nm. Since at 787 nm near infrared light is barely visible, the technique is very patient friendly. The chromophore of AFI is lipofuscin, excessive accumulation of which in lysosomes of RPE cells is a common finding in a variety of hereditary and degenerative retinal disorders[10,11,12]. RPE-lipofuscin seems to derive predominantly from incomplete digestion of photoreceptor outer segments and there is accumulating evidence suggesting an important role of lipofuscin in RPE cell dysfunction and cell loss[16-24]. Both techniques are now often used in a whole range of retinal diseases, including inherited retinal dystrophies, both to detect early signs of disease as well as better and detailed description of the phenotype [10-15].

We provide data on twenty-two molecularly proven PXE patients and twenty-five PXE carriers, which illustrate that infrared and autofluorescence imaging are more sensitive than white light funduscopy and imaging in visualising both early and more evolved signs of PXE retinopathy. These techniques thus represent an important additional tool in the diagnosis and ophthalmic follow-up of PXE families.

Patients and Methods

Twenty-two patients with a clinically (dermatological and ophthalmological examination, skin biopsy) and molecularly (*ABCC6* analysis) confirmed diagnosis of PXE, and twenty-five obligate carriers with one proven *ABCC6* mutation were studied. As such, phenocopies of PXE associated with beta-thalassaemia and PXE-like syndrome with identical retinal characteristics compared with classic PXE were excluded [8,9]. The respective age of patients and carriers ranged from 11 to 68 years (mean 43 years) and 15 to 76 years (mean 44 years). All underwent a complete ophthalmic examination, including assessment of best-corrected visual acuity (BCVA), slitlamp examination, fundoscopy and extensive fundus imaging. Fluorescein and indocyanin green angiography and optical coherence tomography (Stratus OCT, Carl Zeiss Meditec Inc, Dublin, California, USA) were performed when and where required. Ocular ultrasonography (OTI scan Ophthalmic Ultrasound, Ophthalmic Technologies, Toronto, Canada) was used to evaluate (potential) optic disc drusen. Fundus imaging consisted of white light digital photography with a Topcon TRC-50EX fundus camera (Topcon Corporation, Tokyo, Japan). Fundus autofluorescence, red free and infrared monochromatic images were obtained using a confocal scanning laser ophthalmoscope (cSLO; Heidelberg Retina Angiograph HRA2; Heidelberg Engineering, Dossenheim, Germany), the optical and technical principles of which have been described previously[10-15]. The HRA2 cSLO has a small pinhole aperture, suppressing light originating from outside the focal plane to enhance image contrast compared with nonconfocal images. It uses an argon blue laser light with a wavelength of 488 nm for excitation and a barrier filter with a cut off at 500 nm to record fundus autofluorescence and red free imaging. For near infrared imaging, the excitation wavelengths is 787 nm and a barrier filter allows light passage above 810 nm. Full-emission spectra are recorded via a polarization filter to obtain red-free and infrared images. Before acquisition of the autofluorescence sequence, illumination and focus level were adjusted to individual requirements at the infrared mode of the device, in order to generate high quality images. Infrared, autofluorescence and red free images, in series of approximately 50 single pictures per eye, were generated using a 30-degree field-overview mode. The images encompassed the entire macular area, retinal midperiphery and periphery. Following acquisition, any images containing eye movements, blinks or uneven illumination were discarded. After automated alignment and averaging to improve signal to noise ratio by image analysis software (Heidelberg Eye Explorer, Heidelberg engineering, Dossenheim, Germany) mean images were used for further analysis[10]. Finally, HRA 2® software was applied on selected individual and mean images of excellent quality to generate a seamless montage of the entire fundus in AF, RF and IR mode.

All images were analysed for various ocular manifestations of PXE, including angioid streaks, peau d'orange, comets, comet tails and optic disc drusen, in both early, neovascular and advanced atrophic stages of the disease. Autofluorescence and infrared images were compared with the colour pictures. AF was defined as low (hypo-autofluorescence; AF signal lower than background), high (hyperautofluorescence; AF signal higher than background) or unchanged compared to normal background autofluorescence. On infrared images the choroidal vessel contrast is usually negative (dark vessels on light fundus background), the retinal vessels and the optic disc rim appear dark or have dark borders with respect to the surrounding fundus[14].

Informed consent was obtained from all patients and carriers and all procedures were in accordance with the Declaration of Helsinki protocol. The study was approved by the Ethical Committee of the Ghent University Hospital.

Results

Eight male and fifteen female PXE patients were included in this study. BCVA ranged from 12/10 to 1/300. All patient fundi examined revealed at least one characteristic of the PXE retinopathy, which was always present in both eyes of each individual (table 1).

All patients except the youngest patient, a boy of 11 years old, had angioid streaks in both eyes, which could be visualised with colour imaging, RFI, AFI and IRI.

On standard digital colour imaging, streaks could be divided in those with or without adjacent RPE alterations. Streaks lacking bordering RPE abnormalities were seen as the typical brick red coloured streaks, presenting as irregular and jagged lines radiating from a concentric peripapillary ring towards the equator of the eye. In some patients, these were limited to a few almost imperceptible lines (fig. 1) whilst in other patients they presented as thick jagged lines (fig. 2) with an at times complex interlacing network (fig. 3). Angioid streaks were always most prominent at the posterior pole and typically tapered and faded whilst approaching the equator of the eye, with an arborescent pattern of progressively smaller branches. In rare cases (2/22) they continued beyond the equator as irregular, white and depigmented lines, possibly representing calcium deposition in and around the streak (fig. 4).

With infrared imaging, angioid streaks appeared as uniform, well-demarcated dark fissures against a lighter background, even when they passed unnoticed on colour imaging. This classic type of streaks had no visually obvious correlate on AFI, although traces could sometimes be discovered when comparing with IRI (figs. 1 & 6). However, autofluorescence imaging sometimes showed a reticular pattern of hyperautofluorescence in the macular area, remeniscent of patterned hypercalcification of the Bruch membrane in the posterior pole (Fig 1c). Only the youngest of 22 patients did not have angioid streaks (fig. 8).

In all fundi, RPE alterations, mostly loss of pigment and more rarely hyperpigmentation, were observed adjacent to some of the streaks.

RPE hypopigmentation, seen in 26 fundi of 13 patients, yielded a “feathered” appearance to the streak on colour imaging (fig. 5). With AFI, the feathered streaks and surrounding area depleted of pigment, appeared as a triangular area of hypo-autofluorescence, with the base of the triangle oriented towards the optic disc. IR imaging depicted these feathered streaks in a similar way as colour photography did. In older and larger streaks, small islands of normal autofluorescence could be observed within the streak. Such islands in the older, larger streaks are better visible in IRI than on colour pictures. (figs. 7 & 11).

Hyperpigmented RPE alterations were seen as dark brown, round or oval zones of variable size along areas of streaks or in atrophic macular scars in 4 patients. Equal in aspect to atrophic areas, on AFI these appear as hypo-autofluorescent lesions with the same size (Fig. 9). On IRI, a limited hyperintensity was noticed compared to the background was detected. However, this would pass unnoticed on the basis of IR evaluation alone (fig. 9).

Peau d'orange, which, according to some investigators, represents a widespread and patchy increase of pigment in the RPE cells[1,2], could potentially also be due to changes in BM architecture. Indeed, calcification and fragmentation of elastic fibers, which lead to dermal peau d'orange lesions, may also underlie the fundus lesions. The speckled or mottled appearance from the macula to the midperiphery, was seen in 36 (change number after inclusion of new patients) fundi. On colour pictures, this fine mottling was visible in the nasal retina and temporal fundus between the macula and the equator of the eye. On AFI, no difference in autofluorescence was observed in regions of peau d'orange. However, IR imaging always revealed a diffuse speckled pattern of peau d'orange, extending beyond the temporal midperiphery (fig. 8). In 55% of fundi (24/44), the peau d'orange area covered the entire midperiphery to the equator and the posterior pole, including the entire macula. Extensive macular atrophy and/or scarring precluded evaluation of peau d'orange in 10 fundi. The overall extent of peau d'orange, was best appreciated on composite infrared images of the fundus, with often a far larger area considered affected than that visible on white light digital fundus images.

Comets and/or comet tails were observed as solitary, subretinal, nodular, white bodies with a tapered white tail of RPE atrophy extending posterior to the body on colour images in 90% (38/42) of the examined fundi. The body may have some pigmentation at its margin. AFI showed small, punctiform hyperautofluorescent lesions but could not detect all comets individually. IR imaging was able to visualize several comets, which went undetected on colour, as small white hyperintensities. However, the most sensitive technique in detecting those pathognomonic lesions was RFI. With the latter all of these were seen as whitish flecks on a gray background (fig. 10). In some fundi, a spray of comets and comet tails was observed, creating an aspect of "comet rain" (fig. 10).

Optic disc drusen, thought to be composed of hyalin-like calcific material within the substance of the optic nerve head, were seen binocularly in 2 patients and uniocularly in 1 patient using colour imaging and AFI. On standard ophthalmoscopy, buried drusen could be suspected due to an elevated disc with a scalloped margin and without a physiological cup. Surface drusen of the disc appear as waxy pearl-like irregularities. Drusen on the surface of the optic nerve head may be visible with autofluorescence, but if they lie deeper, i.e. on the nerve fibers, the optic disc may look normal. In such cases ultrasonography is indispensable. On AFI, superficial drusen were visualized unambiguously in all examined fundi because of the hyperautofluorescence of the optic disc without attenuation of the signal by overlying tissue (fig. 11). On confirmation B-scan ultrasonography, the drusen were recognized by their high acoustic reflectivity. Optic disc drusen could be suspected on IR imaging and RF imaging afterwards, albeit that they did not stand out in these images.

Fibrovascular scars following choroidal neovascularisation in the macular area were observed in 17 eyes. Autofluorescence in these end-stage fundi was very low in areas of atrophy and high in the junctional zone separating atrophic zones from surrounding, more normal retina. In 5 patients, additional lobular hypo-autofluorescent lesions were noted on AFI, adjacent to but separated from the fibrovascular scars. In 2 patients, previously treated with photodynamic therapy, a paler zone was observed around the area of choroidal neovascularisation on colour imaging (Fig 3). On AFI the borders of a uniform hypo-autofluorescent zone correlate with the borders of the paler zone found around the fibrovascular scar on colour pictures. These paler zones appeared as an atrophic area on IR imaging.

In eight of the twenty-five carriers (3 males and 5 females) examined, comets and comet tails were observed. In 3 carriers the appearance of the comets were similar to those seen in PXE patients. However, in 5 carriers comets were limited to small white dots with neither hyperpigmented borders nor comet tails. No other abnormalities of fundus autofluorescence or infrared imaging, such as limited peau d'orange or angioid streaks were observed.

Case	Age, sex	Eye	BCVA	AS	Pd'O	C(T)	ODD	NV	PDT	Anti-VEGF	*ABCC6* mutations
1	61, F	OD	2/10	+	+	+	-	+	+	-	p.R1459C/-
		OS	9/10	+	+	+	-	+	+	-	
2	43, F	OD	12/10	+	+	+	-	-	-	-	p.R1141X/p.R1141X
		OS	12/10	+	+	+	-	-	-	-	
3	35, M	OD	11/10	+	+	+	-	-	-	-	p.E125K/p.L1025P
		OS	1/300	+	+	+	-	+	+	+	
4	21, M	OD	10/10	+	+	+	-	-	-	-	p.R1141X/p.R1141X
		OS	10/10	+	+	+	+	-	-	-	
5	11, M	OD	10/10	-	+	+	-	-	-	-	c.3506+2T>C/-
		OS	10/10	-	+	+	-	-	-	-	
6	55, F	OD	1/20	+	+	+	-	+	-	-	p.R1141X/p.R1141X
		OS	1.5/10	+	+	+	-	+	-	-	
7	40, M	OD	4/10	+	+	+	-	+	+	-	p.R265G/p.R1141X
		OS	1/10	+	+	+	-	+	+	-	
8	22, F	OD	10/10	+	+	+	-	-	-	-	p.R1141X/p.R1141X
		OS	10/10	+	+	+	-	-	-	-	
9	29, F	OD	10/10	+	+	+	-	-	-	-	p.G1263R/c.4182delGG
		OS	10/10	+	+	+	-	-	-	-	
10	20, M	OD	10/10	+	+	+	+	-	-	-	c.3507-3C>A/p.T944I
		OS	10/10	+	+	+	+	-	-	-	
11	37, F	OD	10/10	+	+	+	+	-	-	-	p.Q154R/-
		OS	9/10	+	+	+	+	-	-	-	
12	66, F	OD	1/20	+	-	-	-	+	-	-	p.R1141X/p.R1141X
		OS	1/10	+	-	-	-	+	-	-	
13	68, F	OD	10/10	+	-	-	-	-	-	-	p.R760Q/p.R1141X
		OS	10/10	+	-	-	-	-	-	-	
14	68, M	OD	2/10	+	-	+	-	+	-	-	p.A1303P/p.L946I
		OS	CF	+	-	+	-	+	-	-	
15	58, F	OD	7/10	+	+	+	-	+	-	+	p.R1141X/c.4103delC
		OS	CF	+	+	+	-	+	+	-	
16	62,M	OD	1/20	+	+	+	-	+	-	+	p.R1141X/p.Q1237X
		OS	CF	+	+	+	-	+	-	-	
17	47, F	OD	10/10	+	-	-	-	-	-	-	c.3506+2T>C/-
		OS	10/10	+	-	-	-	-	-	-	
18	54, F	OD	10/10	+	+	+	-	+	-	+	p.R1141X/p.A1303P
		OS	10/10	+	+	+	-	-	-	-	
19	30, F	OD	10/10	+	+	+	-	-	-	-	p.S398R/c.3364delT
		OS	10/10	+	+	+	-	-	-	-	
20	39, F	OD	12/10	+	+	+	-	-	-	-	p.R1141X/-
		OS	10/10	+	+	+	-	-	-	-	
21	30, F	OD	10/10	+	+	+	-	-	-	-	p.G666R/c.1868-5T>G
		OS	10/10	+	+	+	+	-	-	-	
22	34, M	OD	12/10	+	+	+	-	-	-	-	c.3364delT/p.R518X
		OS	12/10	+	+	+	-	-	-	-	

Table 1

Ophthalmologic characteristics of patients in the present study. BCVA: Best Corrected Visual Acuity, CF: Counting Fingers; AS: Angioid Streaks; Pd'O: Peau d'Orange, C(T): Comet (tails); ODD: Optic Disc Drusen; NV: Neovascularisation; MD: Macular Degeneration; PDT: Photodynamic Therapy; anti-VEGF: anti-Vascular Endothelial Growth Factor antibodies

Discussion

Visualizing the PXE retinopathy with AF and IR imaging

Pseudoxanthoma elasticum manifests with cutaneous, ophthalmological and cardiovascular symptoms, with considerable morbidity[1,2,3]. Because of significant intra- and interfamilial variability in phenotypic severity, making a correct clinical diagnosis of PXE is often difficult, with consequent delay of a potentially essential, early intervention.

This study aimed to evaluate whether novel imaging techniques, such as monochromatic infrared and autofluorescence imaging with cSLO, are able to better visualize the PXE retinopathy compared to standard digital colour fundus pictures, thereby facilitating early diagnosis.

Angioid streaks were observed in all but one fundi, as expected, and could be classified into two types, based on whether or not they were accompanied by surrounding RPE alterations. All eyes contained a mixture of both types. In the absence of RPE alterations, AFI was not able to visualize the streaks - even with multiple contrast settings. In contrast, angioid streaks were very distinct on IR imaging compared to colour fundus photography, and are visible as well-demarcated, dark fissures with high contrast to surrounding tissues. Hence, streaks either absent or initially unnoticed on colour pictures were detected with IRI. Streaks with concomitant RPE alterations showed a feathering pattern, pigmented borders and/or deposits in the middle of the streaks. On AFI, the overall autofluorescence intensity of these feathered streaks appeared lower, which might be explained by loss of lipofuscin in the RPE cells adjacent to the streaks. The pathological substrate of this finding could be absence of photoreceptors, RPE atrophy, RPE proliferation with hyperpigmentation, a decrease of outer segment phagocytosis by RPE cells, or a combination of several of these. Since none of the patients suffered from scotomas in the areas of streaks, which would be expected in case of absent photoreceptors or RPE atrophy, the latter three possibilities seem the most plausible[25]. The hyperpigmented borders appeared as uniform dark hypo-autofluorescent zones with the same size compared to those in the colour images. The decreased autofluorescence could indeed be due to proliferation of the RPE cells at the borders which block AF and to attenuation due to dysfunction of RPE cells overlying the streak which lose their lipofuscin[17]. The presence of dotted areas of normal autofluorescence within longstanding angioid streaks visualised on AF pictures, may represent normal zones of RPE and potentially BM in the center of streaks, due to fragmentation of the main body of the RPE at the streak margin. For these streaks associated with RPE alterations, IR imaging did not reveal any significant difference compared to colour photography.

Media opacities including lens opacification may result in AF images that are of insufficient quality if at all they can be obtained. Such opacities do not hamper IR imaging, due to better penetration of long-wavelength light.

Together, these observations suggest that a combination of AF and IR imaging is essential for optimal visualisation of the different types of angioid streaks and as such is superior to colour imaging for the early detection and follow-up of angioid streaks.

It has been suggested that angioid streaks could disappear in patients with longstanding PXE[26]. We observed streaks becoming obscured by extensive chorioretinal scarring and accompanying proliferation of the retinal pigment epithelium. However, on colour, AF and IR imaging, distinctive

remnants of streaks persisted at the periphery of such lesions, suggesting that angioid streaks are persistent throughout the natural history of the disorder.

Although peau d'orange has previously been described as granular stippling on AFI[26], the pigment alterations seen on colour imaging did not yield any significant difference in fundus autofluorescence in the present study. On infrared imaging, we observed the peau d'orange to be more widespread than originally described, and present throughout the posterior pole and the whole midperiphery. This might be due to pure technical reasons, since IR imaging is less compromised by macular lutein and zeaxanthin pigment. This observation further strengthened our belief that this technique has a significant higher sensitivity in detecting changes of the outer retinal layers compared to colour fundus imaging.

We found IR imaging to be also superior in visualising comets and comet tails in combination with RF imaging. Even comets barely visible or absent on colour pictures could be seen with IRI and RFI. In contrast, on AFI not all comets were recognised. If comets and comet tails indeed are the only pathognomonic ocular characteristic of PXE[7], they are of significant diagnostic value, especially in young patients in whom angioid streaks are often not yet present (fig. 14). Since they can also occur in carriers, a thorough skin evaluation and molecular analysis of the *ABCC6* gene is indicated when such lesions are detected.

Drusen of the optic disc seem to be more common in PXE than in the general population[27]. AFI proved to be efficient in detecting these optic disc drusen which were confirmed on ultrasound. It may be assumed that a common process of abnormal mineralisation might be the predisposing factor both for drusen of the optic nerve head and for comets and comet tails.

In end-stage fundi, increased autofluorescence in the junctional zone around atrophic areas may represent areas of future RPE atrophy[11]. However, these findings are of limited predictive value. The standard examinations to detect subretinal neovascularisation in such patients remain fluorescein and indocyanine green angiography.

Conclusion

Because of extensive variability in clinical severity, the diagnosis of PXE is often delayed until ophthalmological complications occur. Since novel ophthalmic therapeutic agents, such as different agents with anti-VEGF action, have become available, the need for an early diagnosis has become even more important. The present study showed that infrared, red-free and autofluorescent imaging are of significant clinical relevance in PXE, as they contribute to improved imaging quality and hence early diagnosis and more adequate follow-up. Serial, prospective investigations of patients with these techniques can give us important clues as to the natural history of this complex disorder. As such, we consider them part of the standard evaluation of novel and known PXE patients and their family members.

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Figures

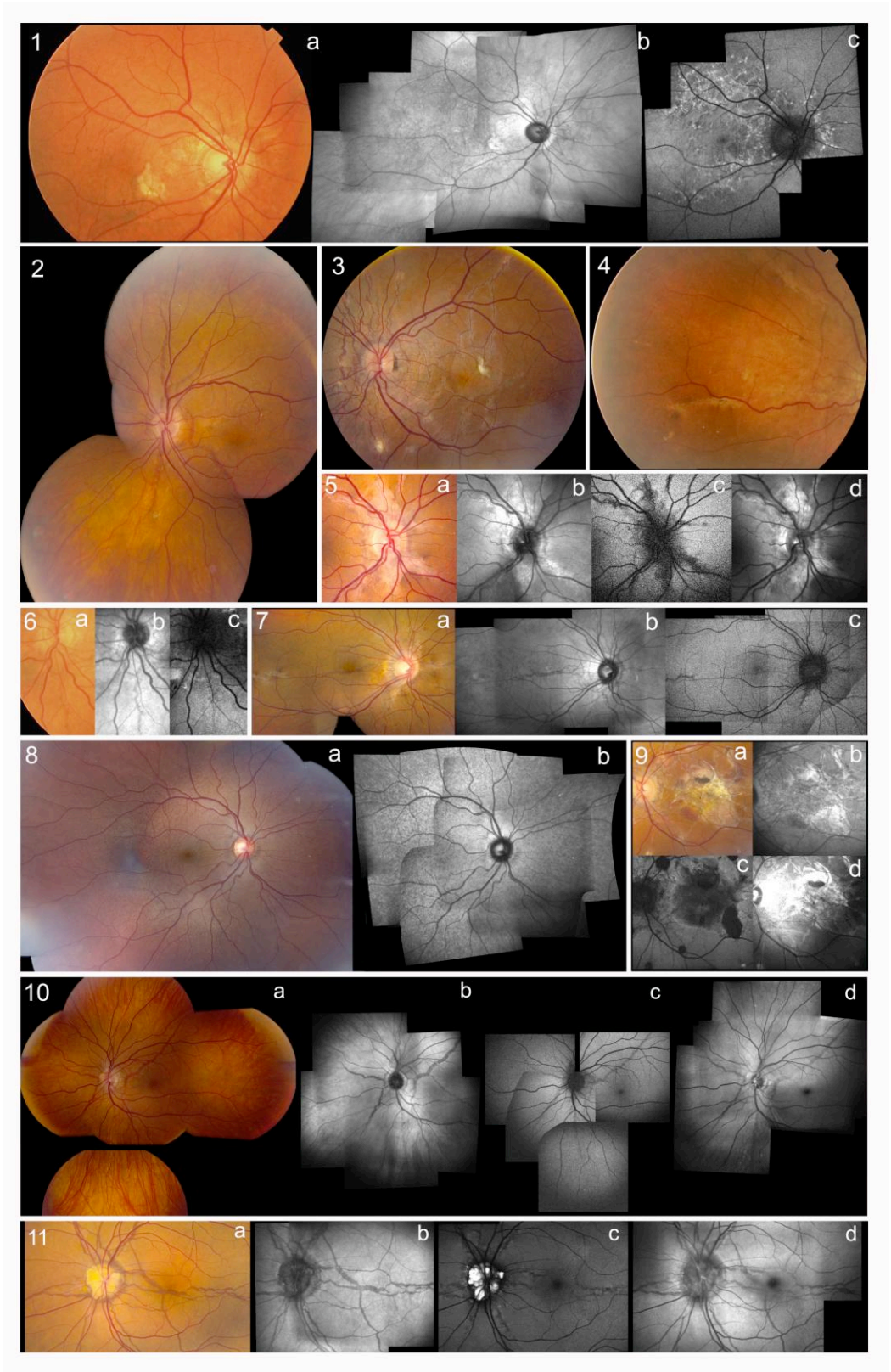

Figure 1
From left to right and top to bottom:

Fig 1: RE of female patient 18, with angioid streaks limited to a few almost imperceptible lines on fundus image radiating from a concentric peripapillary ring towards the equator (a); fine streaks better visible on IRI (b) but not on AFI (c); however, AFI shows reticular pattern of hyperautofluorescence in macular area, reminiscent of patterned hypercalcification of Bruch membrane in posterior pole

Fig 2: RE of female patient 2 with angioid streaks showing as thick jagged lines

Fig 3: Angioid streaks in LE of male patient 3 form complex interlacing network; in addition, atrophy and scar of subretinal neovascular membrane after consecutive treatment with photodynamic therapy and intravitreal bevacizumab are visible in macular area; white lesions around some angioid streaks may represent chorioretinal calcification

Fig 4: Angioid streaks in periphery may become (partially) calcified with whitish aspect as seen in nasal periphery of LE of female patient 6

Fig 5: Aspect of feathered type of angioid streaks in RE of young female patient 8 on white light (a), infrared (b), autofluorescence (c) and red-free imaging (d); note better visibility of the streaks as whitish areas on IRI and RFI than on white light fundus imaging; streaks appear as dark areas on AFI

Fig 6: Parallel white light, infrared and autofluorescence images of infrapapillar angioid streak in LE of female patient 15: whereas streak is better visible on IRI (b) than on fundus image (a), it is hard to see on AFI (c)

Fig 7: RE of female patient 20: small islands of normal retinal tissue within older, broader streaks showed normal autofluorescence, but are better visible in IRI (b) than on colour pictures (a) or AFI (c) or RFI (not shown)

Fig 8: Peau d'orange much more extensively visible on IRI (b) than on colour fundus image (a) in 11-year old male patient 5; note extension of peau d'orange changes around the whole posterior pole on IRI; no angioid streaks present at this early age, although comets and comet tails are visible

Fig 9: Hyperpigmentation due to RPE hyperplasia in macular scar after PDT treatment (a); hyperpigmentation and atrophy appear as dark areas on AFI (c); hyperpigmentation appears as a light zone on IRI (b), and black on RFI (d)

Fig 10: A burst of comets and comet tails inferior to optic disc of female patient 19; note that the comets are best visible on RFI (d) compared to colour fundus imaging (A), IRI (b) and AFI (c); in addition, broad angioid streaks emanating from the optic disc are best seen on IRI (b) and colour fundus image (a)

Fig 11: Superficial optic drusen in LE of patient 11 (a) visualised unambiguously on AFI because of hyperautofluorescence of optic disc (c); disc drusen could be suspected on IRI imaging (b) and RFI imaging (d) albeit that they did not stand out; note areas of normal retinal tissue within broad angioid streaks inferotemporal of fovea

Publication 6

Heterozygous *ABCC6* mutations in ischemic stroke patients: initial experience

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In this pilot study, 206 patients admitted to the Stroke Unit of the Department of Neurology of the Ghent University Hospital because of ischemic stroke were examined both clinically and molecularly in order to detect any previously unnoticed PXE patients or heterozygous mutation carriers. The protocol consisted of:

1/ a careful dermatological examination;

2/ a funduscopic evaluation of the retina using white light;

3/ and molecular analysis of the recurrently mutated *ABCC6* exons (exon 18, 24, 28 and 29), as defined in paper 1.

Although no novel PXE patients could be identified in this stroke population, we were able to show heterozygous *ABCC6* mutations to be significantly associated with ischemic stroke. This exploratory pilot study suggests limited value of systematic clinical evaluation of stroke patients for PXE. Data are however promising in implicating heterozygous *ABCC6* mutations to be associated with sporadic ischemic stroke.

Heterozygous *ABCC6* mutations in ischemic stroke patients: initial experience

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Abstract

Evidence is emerging that ischemic stroke results from the complex interplay between environmental and genetic risk factors. One method to identify genetic factors, is a candidate gene approach. Because of the increased incidence of ischemic stroke in pseudoxanthoma elasticum – an autosomal recessive disease caused by mutations in the *ABCC6* gene – and the higher cardiovascular risk in carriers of one *ABCC6* mutation, we investigated whether heterozygous *ABCC6* mutations are more frequent in ischemic stroke patients compared to the general population.

In 206 consecutive ischemic stroke patients, clinical evaluation could not reveal features of PXE, while *ABCC6* hotspot analysis identified 12 carriers of one *ABCC6* mutation in the stroke group compared to two carriers in a control group of 197 individuals. The calculated Odds Ratio was 6.062 ($p = 0.012$; 95% CI 1.4-24.4).

This exploratory pilot study suggests limited value of systematic clinical evaluation of stroke patients for PXE. Data are however promising in implicating heterozygous *ABCC6* mutations to be associated with sporadic ischemic stroke.

Introduction

Stroke, one of the leading causes of death and long-term disability worldwide, is known to have a heterogeneous etiology. In ischemic stroke – which is the most common form of stroke accounting for over 80% of stroke events – known risk factors (arterial hypertension, dyslipidaemias, diabetes mellitus, cigarette smoking, peripheral artery and cardiac disease) are insufficient to explain stroke risk, making it likely that other causal risk factors and pathways are involved [1,2]. Studies in twins, families and animal models provide substantial evidence for a genetic contribution to ischemic stroke [1]. In recent years, it has become clear that stroke can either occur in the context of monogenic disorders or, more frequently, result from the interplay between modifiable risk factors and genetic susceptibility [2]. Several association studies have described DNA variants, mostly polymorphisms, to be associated with an increased or decreased risk for stroke, while linkage studies identified a number of gene loci possibly involved in stroke [3-12].

One of the monogenic disorders occasionally associated with stroke is pseudoxanthoma elasticum (PXE; OMIM# 264800). This autosomal recessive disorder is characterized by mineralization and fragmentation of elastic fibers and is caused by mutations in the *ABCC6* gene (ATP-binding cassette C6 - OMIM# 603234), encoding an ATP-dependent transmembrane transporter [13]. PXE affects the skin (coalescent yellowish papules in flexural areas of the body), the eyes (retinopathy with angioid streaks, leading to vision loss) and the cardiovascular system (occlusive arterial disease) [14,15]. We have observed a significant increase in the prevalence of ischemic stroke in PXE patients, suggesting that a disabled *ABCC6* pathway may play an important role in stroke pathophysiology [15]. Furthermore, heterozygous carriers of one *ABCC6* mutation (e.g. parents and children of a PXE patient) have been shown to be at a higher risk for coronary and peripheral artery disease, although they do not develop skin features or ocular problems [15,16,17]. Little is known however about a possible association between heterozygous *ABCC6* mutations and sporadic ischemic stroke, although we previously noted PXE carriers to be prone to ischemic stroke [15]. As the carrier frequency of one *ABCC6* mutation in the general population has been estimated to be ~1%, an association with disease could be important for public health [18].

To examine the feasibility and potential relevance of clinical examination for presence of PXE features and molecular analysis of the *ABCC6* gene in ischemic stroke patients, an exploratory pilot study was designed, involving 206 consecutive patients with confirmed ischemic stroke who were admitted to the Stroke Unit of the Ghent University Hospital. While not being able to detect true PXE patients in this cohort, we demonstrated heterozygosity for *ABCC6* mutations to be more prevalent in an ischemic stroke population, compared to the general population.

Patients and Methods

Patient selection

Two-hundred and six consecutive patients admitted to the Stroke Unit of the Ghent University Hospital between March 2004 and August 2007 with a proven diagnosis of ischemic stroke were approached for written consent to participate in the study. In case of will inaptitude, the closest relatives gave informed consent. Patients with cerebral haemorrhage or cerebral venous thrombosis were not included.

Baseline demographic data (age, sex) and history of conventional risk factors (hypertension, diabetes, hypercholesterolaemia, current smoker, peripheral and coronary vascular disease, valvular disease) were obtained (table 1).

	Cases (n=206)	Controls (n=197)	p-value
Mean age (± SD)	69 (± 11,5)	67 (± 11,5)	0,08
Sex (M:F ratio)	1,45	1,01	0,07
Hypertension (%)*,‡	150 (73)	0	<0,0001
Diabetes Mellitus (%)	49 (23)	34 (17)	0,11
Dyslipidaemia (%)‡	72 (35)	18 (9)	0,17
Smoking (%)	18 (7)	10 (5)	0,015
Heart valve disease‡	7 (3)	0	<0,0001
Coronary disease‡	56 (27)	0	<0,0001
Peripheral vascular disease‡	35 (17)	0	<0.0001

Table 1

Demographic characteristics of cases and controls. *Hypertension defined as bloodpressure > 160/90 or history of hypertension or hypertensive treatment ; † History of diabetes or confirmed laboratory diagnosis; ‡ Significant differences ($p<0.05$) between cases and controls

On admission for their stroke a detailed medical history, including vascular risk factors and previous treatments, was obtained in all patients. All patients had a complete clinical evaluation and underwent a full cardiovascular examination, including ECG, chest X-ray and routine blood analyses, Doppler sonography of the extracranial arteries, 24-h electrocardiogram monitoring and transthoracic echography of the heart. CT scan of the brain was performed on admission after stroke and repeated within 2 weeks. Transoesophageal echocardiography, cerebral MRI and MR angiography and/or conventional angiography were carried out at the discretion of the clinician. The stroke types were classified according to the Trial of ORG 10172 in Acute Stroke Treatment (TOAST) criteria (table 2) [19]. All patients underwent thorough skin inspection of the flexural areas and lip mucosa to assess possible skin and/or mucosal lesions and funduscopy to evaluate the presence of a PXE retinopathy.

The control population consisted of a group of 197 Belgian individuals, age-matched with the study group, undergoing a routine check-up and presenting without known cardiovascular events. A blood sample was obtained from patients and controls for molecular analysis of the *ABCC6* gene.

This study was approved by the Ethical Committee of the Ghent University Hospital and the Declaration of Helsinki Principles was followed.

Molecular analysis of the *ABCC6* gene

Molecular analysis of the *ABCC6* gene was limited to the most prevalent mutations, i.e. the p.R1141X nonsense mutation (c.3421C>T), the multi-exon deletion spanning exons 23-29, as well as four previously defined recurrently mutated exons (exon 18, 24, 28 and 29) [15]. We showed previously that this detection strategy allows to identify 75% of all *ABCC6* mutations in a Belgian population, thus being representative for the whole *ABCC6* mutation spectrum [15].

Genomic DNA was isolated from whole blood (QIAamp blood kit, Qiagen®) according to an established procedure. The *ABCC6* coding region was amplified using previously described PCR primers [20].

For the detection of the exon 23-29 deletion, primers were used as described by Le Saux et al. [13].

The coding region and intron/exon boundaries of *ABCC6* were analyzed through direct sequencing using an Applied Biosystems 3730xl Sequencer®, with ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems®). Unreported sequence variants were defined as causative according to criteria reported by Cotton and Scriver, 1998. Nucleotide numbers are derived from gDNA *ABCC6* sequences (GenBank accession no. NM_001171)

Statistical analysis

Differences in allelic frequencies between patients and controls were examined using the two-tailed Fisher's Exact test. Logistic regression analysis was used to assess modification of mutation-stroke interaction by cardiovascular risk factors (hypertension, dyslipidaemia, diabetes mellitus, and smoking). Results were expressed as odds ratios (OR) and their 95% confidence intervals (CI). An OR of<1 corresponds to a reduced risk of disease; an OR of >1 with an increased risk. The significant level was set at α=0.05 two-tailed.

Results

The study group consisted of 206 consecutive unrelated patients admitted with ischemic stroke (122 men, 84 women; mean age 69 ± 11.5 years) and 197 control individuals (99 men, 98 women, mean age 67 ± 11.5 years). Distribution of subtypes of stroke is presented in table 2. Most cases were due to large vessel disease or cardio-embolic strokes.

Subtype	All stroke samples	
	Number	%
Large Vessel Disease (LVD)	96	47
Small Vessel Disease (SVD)	41	20
Cardio-embolic Stroke	49	23
Other/Unclear	20	10
TOTAL	206	100

Table 2

Different stroke subtypes based on TOAST classification

Thorough skin and ophthalmological evaluation was not able to reveal characteristic skin lesions or features of the PXE retinopathy in any of the examined patients. Subsequently, we analyzed the previously identified recurrently mutated exons and evaluated the presence of the multi-exon deletion spanning exons 23 through 29. A total of 12 mutations was identified, all of which were in the heterozygous state. Two mutations were recurrent, i.e. the frequent p.R1141X nonsense mutation was found in 2 patients, and the multi-exon 23-29 deletion was present in 3 patients (table 3). The other mutations were unique and included 1 previously reported splice site mutation, 1 deletion and 5 missense mutations, 2 of which were novel (p.E1376A, p.I1345T). All missense mutations altered amino acids that are highly conserved among orthologs (Mus musculus, Rattus norvegicus, Danio rerio and Fugu rubripes) and conserved in paralogs. They were not found in a panel of 200 control individuals. In the stroke population, seven mutations were located within exon 29 or caused deletion of this exon. The remainder were located in exon 24 (p.R1141X) or 28 (p.I1345T). No mutations were detected in exon 18.

Pat.	SEX	Current age (yrs)	Stroke Type	N	Age at first Event (yrs)	Cardiovascular risk factors	FH	Mutation*	
005	F	71	SVD	2	?	-	-	c.4208+9G>A	-
006	F	61	SVD	1	61	Hyperhomocysteïnaemia	+	Del23-29	-
026	M	74	LVD	2	74	HCh (R/)	-	c.4104delC	-
035	M	67	CE	1	67	HCh (R/)	-	c.3421C>T	p.R1141X
037	F	79	LVD	1	79	AHT (R/), DM (R/)	+	c.4127A>C	p.E1376A
065	F	43	LVD	M	?	-	+	c.4127A>G	p.E1376G
088	M	64	LVD	1	64	-	-	Del23-29	-
098	M	78	SVD	M	?	AHT (R/); HCh (R/), tabagism	-	c.4168G>A	p.E1400K
135	F	63	LVD	1	63	-	-	c.3421C>T	p.R1141X
143	F	53	LVD	1	53	DM, HCh (R/)	-	c.4082A>G	p.D1361G
146	F	74	CE	2	59	DM (R/)	-	c.4043T>C	p.I1345T
166	M	71	CE	1	71	AHT, tabagism	-	Del23-29	-

Table 3

Clinico-molecular characteristics of the 12 stroke patients carrying an *ABCC6* mutation. N: Number of stroke events; FH: Familial history; SVD: Small vessel disease; LVD: Large vessel disease; CE: Cardio-embolic stroke; M: Multiple; HCh: Hypercholesterolaemia; R/: Adequately treated; AHT: Arterial hypertension; DM: Diabetes mellitus *GenBank accession no. NM_001171.2. For cDNA numbering +1 corresponds to the A of the ATG translation initiation codon

Molecular analysis of the control population revealed a mutation (p.R1141X) in only two individuals (aged 52 and 64). The calculated OR for the presence of an *ABCC6* mutation was 6.062 (p=0.012; 95% CI 1.4-24.4). Logistic regression analysis for cardiovascular risk factors (arterial hypertension, dyslipidaemia, diabetes mellitus, smoking, peripheral artery and cardiac disease) as modifiers of the interaction between the detected mutations and stroke did not yield significant results.

Besides these causal mutations, several *ABCC6* polymorphisms were detected, most of which were intronic and were predicted not to affect splicing. Of the exonic polymorphic variants, p.Q1390E (c.4168C>G; exon 29) was found in 3 patients.

The clinical characteristics of the stroke patients in which a heterozygous *ABCC6* mutation was found are listed in table 3. The mean age of these patients (7 female and 5 male) was 66 years (range 43-79 years). Six patients (50%) presented with large vessel disease. The remaining patients suffered from small vessel disease (25%) of cardio-embolic stroke (25%). About half of the patients had a history of one or more previous cerebrovascular disease episodes. A positive familial history of stroke was found in only 3 patients.

Discussion

Ischemic stroke is observed in a number of monogenic disorders, one of which is PXE. However, the majority of strokes have a multifactorial etiology. Strong evidence from epidemiological and animal studies have implicated genetic influences in the pathogenesis of ischemic stroke. The identification of individual causative mutations is difficult, in part because genetic influences are likely to be of polygenic nature. In the present study, we have used a candidate gene approach to investigate the contribution of genetic factors in ischemic stroke. Indeed, we observed a remarkable increase in ischemic stroke incidence in Belgian PXE patients compared to the general population [15]. As heterozygous carriers of 1 *ABCC6* mutation have been suggested to have an increased risk for cardiovascular events, we investigated whether the incidence of PXE patients with a mild phenotype or heterozygous carriers of PXE is increased in an ischemic stroke population.

Despite thorough dermatological and ophthalmological screening of all 206 patients, we were not able to detect mild or previously unnoted PXE features. Previously, four young PXE patients have been described in whom the presenting complaints were cardiovascular complications [23]. Although the mean age in this study population is significantly higher, our findings do not demonstrate an increase of (homozygous) PXE patients with a mild phenotype among the cerebrovascular patient cohort. Nevertheless, it remains of interest to perform a brief and inexpensive skin and fundoscopic examination, particularly in (young) individuals suffering from ischemic stroke without other predisposing vascular risk factors.

However, molecular analysis of the *ABCC6* gene revealed a heterozygous mutation in 12 patients (6 %). As the estimated carrier frequency of *ABCC6* mutations in the general population is approximately 1%, this result indicates that heterozygosity for an *ABCC6* mutation is a risk factor for ischemic stroke with an odds ratio of 6.023. Other risk factors, such as smoking, hypertension, diabetes mellitus or dyslipidaemia, did not significantly modify the interaction between the mutations and the stroke episode. Considering that the detection rate of mutations with our molecular protocol is 75%, it can be postulated that approximately one quarter more mutations (i.c. 3 mutations) would have been found if the whole gene would have been sequenced. This would increase the proportion of *ABCC6* carriers to 7,2% (OR = 7.6; p=0.002; 95%CI: 1.9-30.5). One recurrent polymorphism was found in three stroke patients. Although this polymorphism resides within the coding sequence of the second nucleotide binding fold (NBF2), known to be critical for ATP-binding there is no evidence to suggest that this base pair variant would have a functional effect on ABCC6 transport function.

The stroke phenotypes which are associated with these *ABCC6* mutations are diverse, including mostly large vessel disease but also small vessel disease and cardio-embolic stroke. This distribution – with large vessel disease being the main stroke type – concurs with our previous observations in PXE patients [15]. This heterogeneity of stroke phenotypes found to be associated with *ABCC6* mutations, renders it difficult to delineate a specific subgroup of ischemic stroke patients whom might be prone to *ABCC6* mutations. Thus, applying this molecular analysis in routine diagnostics is precocious at this time. Importantly, a negative familial history should not lead to the conclusion that a genetic influence is unlikely. Several of the patients studied had additional vascular risk factors for stroke, underscoring that stroke is a multifactorial disorder, in which an *ABCC6* mutation may act as a genetic susceptibility factor.

Our findings concur with the previously suggested increased cardiovascular risk in heterozygous carriers of *ABCC6* mutations and implicate yet another member of the superfamily of ABC transporters in the diseased brain. Besides the role of these ATP-dependent proteins in drug resistance (e.g. in intractable epilepsy), certain members have been described in relation to ischemic stroke [24-26]. ABCB1 expression was found to be elevated after stroke, having a deleterious effect on neuroprotective agents [25]. Conversely, two polymorphisms in *ABCA1* – the causal gene for Tangier disease – were found to be associated with a decreased risk for ischemic stroke [26].

The pilot nature of this study has some limitations. First, the study was done with a relatively small sample size, among others because of the intensive character of the physical examinations. This sample size may attribute for the large 95% confidence interval which is observed. Second, analysis of recurrent mutations as an initial screening method for the *ABCC6* gene, leaves the issue of whether more mutations can be found by performing the full analysis of the coding region. These study limitations not withstanding, our data strongly suggest that it is not useful to routinely perform physical evaluation of ischemic stroke patients for PXE. Our findings appear to be promising in implicating heterozygous *ABCC6* mutations in non-syndromic stroke,

providing a basis for future studies to validate these results. Such studies should investigate the whole coding region of the *ABCC6* gene – combining direct sequencing and techniques focussed on detecting deletions such as MLPA – in large study cohorts, preferentially combining different ethnic backgrounds.

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3.3 Identification and etiopathogenetic study of a PXE related disorder: unravelling a common final pathway with PXE

Publication 7

Pseudoxanthoma elasticum-like phenotype with cutis laxa and multiple coagulation factor deficiency represents a separate entity.

Olivier M. Vanakker, Ludovic Martin, Dealba Gheduzzi, Bart P. Leroy, Bart Loeys, Veronica I.Guerci, Dirk Matthys, Sharon F. Terry, Paul J. Coucke, Ivonne Pasquali-Ronchetti, Anne De Paepe.

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This papers gives a detailed account of the identification of a novel disorder in which patients initially present with symptoms and signs typical for PXE. During the course of the disorder, a generalized increase of inelastic skin folds is observed, resembling cutis laxa and hence highly atypical for PXE. The clinical differences with PXE – among which are a mild retinopathy and the consistent presence of a clotting deficiency – are discussed, as well as the particular electronmicroscopical features of this disease.

Through a candidate gene approach, we were able to identify the causal gene for this disorder as the *GGCX* gene, coding for an important enzyme in the vitamin K-cycle. These data gave important insights in the pathogenesis of this PXE-like disorder – implicating vitamin K-dependent inhibitors of calcification – and would prove to be of great value to gain insights in the final pathway leading to PXE (publication 8).

Pseudoxanthoma Elasticum-Like Phenotype with Cutis Laxa and Multiple Coagulation Factor Deficiency Represents a Separate Genetic Entity

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Data on six patients with a Pseudoxanthoma Elasticum (PXE)-like phenotype, characterized by excessive skin folding (resembling cutis laxa) and a deficiency of the vitamin K-dependent clotting factors (II, VII, IX, and X) are presented. A comparison is made between the clinical, ultrastructural, and molecular findings in these patients and those seen in classic PXE and cutis laxa, respectively. Clinical overlap with PXE is obvious from the skin manifestations of yellowish papules or leathery plaques with dot-like depressions at presentation, angioid streaks and/or ocular peau d'orange, and fragmentation and calcification of elastic fibers in the dermis. Important phenotypic differences with PXE include much more severe skin laxity with spreading toward the trunk and limbs with thick, leathery skin folds rather than confinement to flexural areas, and no decrease in visual acuity. Moreover, detailed electron microscopic analyses revealed that alterations of elastic fibers as well as their mineralization were slightly different from those in classic PXE. Molecular analysis revealed neither causal mutations in the *ABCC6* gene (ATP-binding cassette subfamily C member 6), which is responsible for PXE, nor in *VKORC1* (vitamin K 2,3 epoxide reductase), known to be involved in vitamin K-dependent factor deficiency. However, the *GGCX* gene (gamma-glutamyl carboxylase), encoding an enzyme important for γ-carboxylation of gla-proteins, harbored mutations in six out of seven patients analyzed. These findings all support the hypothesis that the disorder indeed represents a separate clinical and genetic entity, the molecular background of which remains to be unraveled.

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INTRODUCTION

Pseudoxanthoma Elasticum (PXE – OMIM no. 264800) is an autosomal-recessive multisystem disease, affecting mainly the eyes (peau d'orange (Pd'O), angioid streaks (AS), loss of central vision owing to neovascularization and subsequent exudation, and hemorrhage), the skin (yellowish papules or plaques of coalesced papules on the neck and in flexural areas), and the cardiovascular system (diffuse occlusive arterial disease). In some patients, the skin lesions can be progressive, evolving toward excessive skin folding in the flexural areas (McKusick, 1966; Neldner, 1988; Hu *et al.*, 2003; Chassaing *et al.*, 2005). Histologically, it is characterized by fragmentation and mineralization of the elastic fibers in the reticular dermis.

PXE is caused by mutations in the *ABCC6* gene (ATP-binding cassette subfamily C member 6 – chromosome 16p13.1) encoding an ATP-dependent transmembrane transporter whose substrate and pathophysiological role remain to be elucidated (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000; Struk *et al.*, 2000).

Cutis laxa (OMIM nos. 123700; 219100) is a heterogeneous group of acquired or inherited (autosomal-recessive or -dominant) disorders, characterized by loose, sagging, and redundant skin. The clinical findings can be limited to the skin, although extracutaneous manifestations (pulmonary emphysema, hernias, intestinal diverticuli, ocular anterior segment abnormalities) have also been described (Lewis *et al.*, 2004; Ringpfeil, 2005).

The histopathology of cutis laxa, using van Giesson stains, can reveal both loss and fragmentation of elastic fibers in the reticular dermis (Lewis *et al.*, 2004; Ringpfeil, 2005). Although the molecular background of cutis laxa is largely

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Abbreviations: ABCC6, ATP-binding cassette subfamily C member 6; AS, angioid streaks; GGCX, gamma-glutamyl carboxylase; Pd'O, peau d'orange; PXE, Pseudoxanthoma Elasticum; VKCFD1/2, congenital deficiency of the vitamin K-dependent factors; VKORC1, vitamin K 2,3 epoxide reductase

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unknown, causal mutations have been reported in the elastin gene (*ELN* on chromosome 7q11.2) and in the fibulin-5 gene (*FBLN5* on chromosome 14q32.1) (Tassabehji *et al.*, 1998; Loeys *et al.*, 2002; Markova *et al.*, 2003).

We describe six patients in whom a clinical diagnosis of PXE was initially made and subsequently supported by light microscopy on skin biopsy. However, these patients progressively developed excessive skin folds which, although initially confined to the flexural areas, spread both toward the abdomen and limbs. Moreover, all six patients were also found to have a deficiency of the vitamin K-dependent coagulation factors (factors II, VII, IX, X). Two patients had a history of or suggestive of cerebral aneurysms.

This phenotype has been previously described in a few case reports (MacMillan and Vickers, 1971; Rongioletti *et al.*, 1989; Le Corvaisier-Pieto *et al.*, 1996) but to our knowledge, no molecular data are available.

We have undertaken a detailed electron microscopy study of the ultrastructural characteristics of the dermis of these patients. Additionally, a molecular study of the *ABCC6* gene was performed as well as an initial candidate gene screening for the clotting disorder.

Based on the clinical, structural, and molecular findings on these six novel patients, we hypothesize that their condition represents a separate genetic entity.

RESULTS

Clinical description

Six Caucasian patients with initial signs of typical yellowish papules that coalesce and form plaques with regularly depressed dots, sometimes referred to as cutaneous ''Pd'O'' (Figure 1b), developed an increasing number of excessive, leathery skin folds, which gradually spread beyond the flexural areas of the body (Figure 1c–f). The papular and leathery skin lesions with dot-like depressions, however, remained stable. Fundoscopy revealed only limited AS and/or Pd'O without comets temporal to the macula in patients 1 through 4 and normal visual acuity in all patients (Figure 1a). During follow-up, both fundus appearance and visual acuity remained unchanged.

Cardiovascular investigations, including clinical examination, electrocardiography, and ultrasonography, showed subclinical atherosclerotic plaques in the lower limbs in two patients and vascular occlusion with intermittent claudication in one patient. In patient 1, two cerebral aneurysms were subsequently discovered at age 36 and 46 years and successfully treated.

Routine blood coagulation tests revealed a prolonged prothrombin time with decreased levels of the vitamin K-dependent clotting factors (Table 1). Clinical manifestations were present in only two patients. Patient 3 suffered three meningeal hemorrhages over a period of 20 years. Further bleeding events include a postpartum hemorrhage after delivery of her only child and one episode of unexplained hematemesis. Patient 5 had a history of epistaxis, spontaneous gingival bleeding, and severe vaginal hemorrhages.

Details on the individual phenotypes can be found in Table 2.

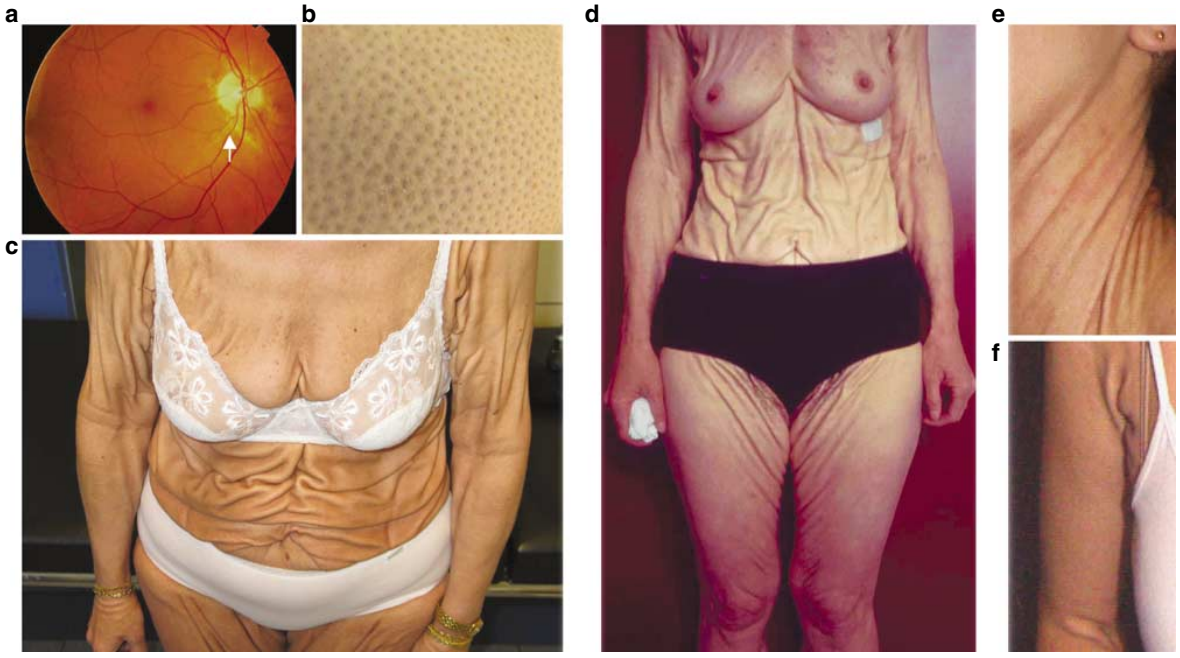

Figure 1. Clinical characteristics of the presented phenotype in several patients. Note (**a**) a mild retinopathy, (**b**) skin lesions featuring yellowish papules that coalesce and form plaques with regularly depressed dots, and (**c**–**f**) generalized excessive and leathery skin folds.

Table 1. Biochemical analysis results of the PT and vitamin K-dependent coagulation factor activity levels

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Normal
PT (INR)	1.81	1.84	1.97	2.19	1.7	1.9	0.8–1.2
Factor II (% nl)[1]	66	61	38	38	20	18	100 (90–150)
Factor VII (% nl)	26	39	50	62	74	88	100 (90–150)
Factor IX (% nl)	70	71	103	90	48	56	100 (90–150)
Factor X (% nl)	15	40	20	17	20	18	100 (90–150)

PT, prothrombin time; INR, International Normalized Ratio.
[1]Percentage of normal activity.

Table 2. Comparison of the phenotypical, histopathological, and molecular characteristics of the six presented patients (1–6) and the four previously reported cases (7–10)

	1	2	3	4	5	6	7	8	9	10
Sex	F	F	F	F	F	M	M	M	F	F
Age[1]	46	47	67	32	46	44	40	33	24	24
Age of onset skin symptoms[1]	18	13	3	18	NA	NA	16	Puberty	Puberty	Puberty
Positive familial history	–	–	–	–	NA	NA	–	–	+	+
Generalized skin folds/laxity	+	+	+	+	+	+	+	+	+	+
Yellowish papules	–	+	+	+	+	+	NA	NA	–	–
Dot-like depressions	+	+	+	+	+	+	NA	NA	–	–
Yellow mucosal pattern	–	+	+	–	NA	NA	NA	NA	NA	NA
Esthetic surgery performed	–	+	+	+	NA	NA	NA	NA	NA	NA
Positive calcium stain	+	+	+	+	+	+	+	+	+	+
AS	+	+	+	+	–	–	–	–	–	–
Ocular Pd'O	–	+	–	+	–	–	NA	–	–	–
Decreased visual acuity	–	–	–	–	–	–	–	–	–	–
Clotting deficiency	+	+	+	+	+	+	+	+	+	+
Abnormal bleeding tendency	–	–	+	–	+	–	+	–	+	+
Subclinical atherosclerosis	–	+	+	–	+	–	–	–	+	+
Weak peripheral pulsations	–	–	–	+	–	–	NA	NA	NA	NA
Cerebral aneurysms	+	–	?	–	NA	NA	–	–	–	–
ABCC6 mutations	–	–	–	–	NA	NA	–	NA	NA	NA
VKORC1 mutations	–	–	–	–	–	–	–	NA	NA	NA

AS, angioid streaks; F, female; M, male; NA, not available; Pd'O, peau d'orange.
[1]In years.
Case 7: Le Corvaisier-Pieto *et al.*, 1996; case 8: Rongioletti *et al.*, 1989; cases 9 and 10: MacMillan and Vickers, 1971.

Ultrastructural findings

By light microscopy, the overall appearance of the dermis was identical to that typical of PXE. The reticular dermis presented areas in which elastic fibers were polymorphous, fragmented, and mineralized, as shown by Von Kossa staining.

At the ultrastructural level, the alterations of the elastic fibers could be better analyzed and compared with those of PXE and revealed peculiar features. Although the two classical types of mineral precipitates, fine granular and bulky calcifications, were present, elastic fibers had a more fragmented and mottled appearance compared to those typical of PXE. In longitudinal sections, elastic fibers appeared to be made of loose, thin strands of polymorphous elastin material. Moreover, very often mineralization occupied only a limited area within huge elastic fibers and was organized as peculiar small electron-dense crystal-like precipitates. Several collagen fibrils were fused forming the so-called ''collagen flowers'' (Figure 2).

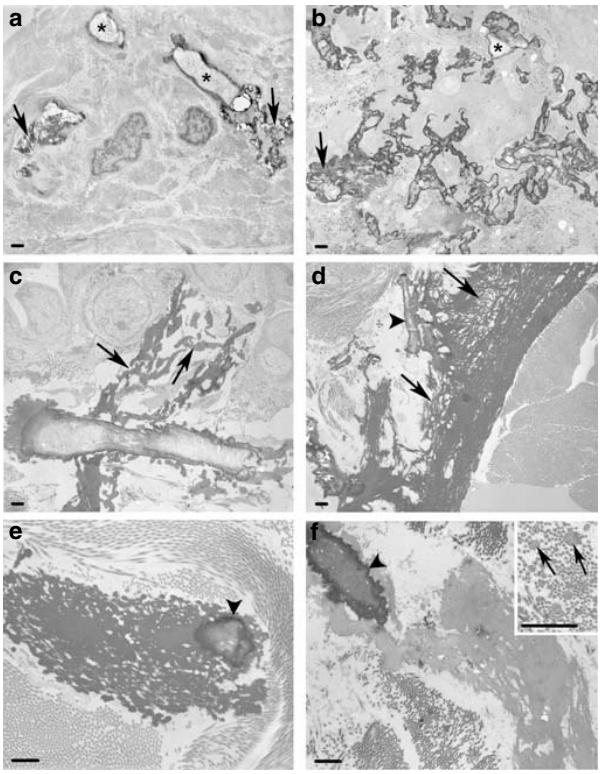

Figure 2. Ultrastructural characteristics of the presented phenotype. At low magnification electron microscopy, dermal alterations are mainly located in the reticular dermis and appear very similar to those in PXE. The great majority of elastic fibers are polymorphous, fragmented, and mineralized and are surrounded by rather thick and compact collagen bundles (**a** and **b**). Both the fine granular (**a** and **b**, asterisks) and the bulky types (**a** and **b**, arrows) of elastic fiber mineralization, always described in PXE, are present. However, different from classical PXE, the great majority of the apparently normal elastic fibers are organized as rather thin and polymorphous strands of elastin material connected into a loose network (**c** and **d**, arrows). Moreover, calcification affects often the marginal areas of elastic fibers (**d**–**f**, arrowheads), whereas in PXE mineralized fine deposits are always in the core of elastic fibers. Similar to PXE, collagen fibrils can be fused forming the so called ''collagen flowers'' (**f**, inset, arrows). Bar = 1 μm.

Molecular results

Molecular analysis of the *ABCC6* gene, responsible for PXE, failed to identify mutations in all six patients, and in one patient reported earlier (Le Corvaisier-Pieto *et al.*, 1996).

Sequencing of the *VKORC1* gene (vitamin K 2,3 epoxide reductase), responsible for vitamin K recycling, did not reveal any mutation. Analysis of the second gene involved in the clotting disorder, *GGCX* (gamma-glutamyl carboxylase), revealed six novel missense mutations and one novel nonsense mutation in the seven patients, including the patient reported earlier (Table 3). Mutations occurred in homozygous or compound heterozygous form in four patients. In two other patients, only one mutation could be detected. These mutations affected amino acids which were fully or almost fully conserved in *Mus musculus*, *Rattus norvegicus*, *Fugu rubripes*, *Anopheles*, and *Drosophila*. None of these mutations were found in 200 control chromosomes of unrelated healthy Europeans.

Table 3. Mutations found in the six presented cases (1–6) and one earlier reported case (7)

Patient	Allele 1		Exon	Allele 2		Exon
1	p.W493S	c.1506G>C	12	p.W493S	c.1506G>C	12
2	—	—	—	—	—	—
3	p.R476C	c.1454C>T	10	—	—	—
4	p.R476H	c.1455G>A	10	—	—	—
5	p.Q374X	c.1149C>T	8	p.G537Y	c.1339G>T	12
6	p.Q374X	c.1149C>T	8	p.G537Y	c.1339G>T	12
7	p.F299S	c.924T>C	8	p.G558R	c.1700G>A	12

DISCUSSION

The six patients described show clinical overlap with PXE, hence the initial diagnosis of a severe form of that disorder. All initially manifested skin manifestations considered typical of PXE, such as yellowish papules and/or plaques with dot-like depressions characterized by light microscopy as fragmentation and calcification of elastic fibers in the reticular dermis. Moreover, the observed retinopathy (AS originating from the optic disc, Pd'O lesions temporal to the macula) in these patients was anatomically similar if not identical to that seen in PXE.

However, clinical, ultrastructural, and molecular differences allow us to discriminate the phenotype of these patients from PXE and from cutis laxa (Table 4).

First, these patients are remarkable for the severity of their skin involvement. Increased skin laxity and excessive skin folding can be seen in PXE, but usually these features remain confined to the flexural areas of the body (McKusick, 1966; Neldner, 1988; Hu *et al.*, 2003; Chassaing *et al.*, 2005). In contrast, in these patients, the leathery skin folds extended toward the limbs and abdomen leaving only the face, hands, and feet unaffected. Interestingly, the speed of evolution of the skin laxity was quite different between patients. Whereas progression over several years in patients 1 and 4 led to the very severe skin phenotype in their early thirties, patient 3 already suffered from severe skin laxity during her childhood, and patient 2 manifested rapid progression during puberty.

Secondly, the fundus examination revealed only limited AS and/or mild Pd'O. Moreover, visual acuity remained stable in all four patients during follow-up. In contrast, in PXE, up to 60% of patients suffer from considerable loss of central vision. Although sample size is small, this suggests that in this PXE-like phenotype, the retinopathy is milder and has a more benign prognosis.

Third, electron microscopy revealed that dermis alterations, especially those of elastic fibers, were similar, but not identical, to those in PXE. Similarities with PXE were that (i) changes occurred in the same areas of the dermis; (ii) not

Table 4. Clinical, ultrastructural, and molecular characteristics of the PXE-like syndrome with a coagulation disorder *versus* PXE and cutis laxa

	PXE-like	PXE	Cutis laxa
Generalized skin folds/laxity	Always present	Not present	Often present
Positive Von Kossa stain of the dermis	Present	Present	Not present
Electron microscopy of the dermis	Mineralized elastic fibers	Mineralized elastic fibers	Scarce and mottled elastic fibers
Retinopathy (AS/Pd'O)	Present but mild	Present and often severe	Not present
Decreased visual acuity	Not present	60%	Infrequent
Clotting deficiency	Always present	Not described	Not described
Atherosclerosis	Subclinical in 50%	(Sub)clinical in 55%	Infrequent
Cerebral aneurysms	Present	Infrequent	Infrequent
Abnormal bleeding tendency	Present (50%)	Infrequent (10%)	Not described
ABCC6 mutations	Not present	Present (DR=96%)	Not present

AS/Pd'O, angioid streaks/peau d'orange; DR, detection ratio; PXE, Pseudoxanthoma Elasticum.

all elastic fibers were mineralized, but in those that were, the two types of mineralization already described in PXE were present; (iii) fibroblasts had huge cisternae of the endoplasmic reticulum; (iv) lateral fusion of collagen fibrils were observed in one patient. However, elastic fibers, either mineralized or not, were different from that typical of PXE. In patients 1, 2, and 3, they were often made of aggregates of distinct strands of elastin and mineralization was mostly confined to the periphery of the fibers (case 1) or was associated to the most compact areas of the fibers (cases 2 and 3). In contrast, in patient 4 elastic fibers were huge and compact and mineralization was very severe. Moreover, electron-dense crystal-like bodies, never observed in PXE, were present in all patients in the central core of finely mineralized elastic fibers.

Finally, molecular analysis of the *ABCC6* gene, responsible for PXE, failed to identify mutations in all of the patients, including the one reported earlier.

Classical cutis laxa syndromes could also be excluded because neither the retinopathy nor mineralization of elastic fibers is seen in those disorders (Krill and Archer, 1972; Lewis *et al.*, 2004).

A second feature of the phenotype is a deficiency of the clotting factors II, VII, IX, and X, which are synthesized in the liver and depend on vitamin K for their function. Such deficiency can either be acquired or congenital (Oldenburg *et al.*, 2000; Sadler, 2004; Zhang and Ginsburg, 2004). However, in our patients there was no evidence for an acquired form, as they did not exhibit any hepatic disease or malabsorption.

Congenital deficiency of the vitamin K-dependent factors (VKCFD) is a very rare autosomal-recessive disorder. It is caused either by mutations in the *GGCX* gene (VKCFD1 – OMIM no. 277450) on chromosome 2p12, or in the *VKORC1* gene (VKCFD2 – OMIM no. 607473) on chromosome 16p11.2. These genes encode a vitamin K-dependent carboxylase and vitamin K 2,3 epoxide reductase, respectively (Oldenburg *et al.*, 2000; Li *et al.*, 2004; Rost *et al.*, 2004a, b; Zhang and Ginsburg, 2004). Both enzymes are essential for post-translational γ-carboxylation of clotting factors, enabling them to attach to the phospholipid bilayer of membranes as an essential prerequisite for blood coagulation (Zhang and Ginsburg, 2004). The clinical features of VKCFD1 and -2 are highly variable and may include epistaxis, (neonatal) intracranial hemorrhage, hemarthrosis, etc., although several patients remain asymptomatic (Zhang and Ginsburg, 2004). Significant bleeding diathesis was observed in cases 3 and 5, although it remains uncertain whether this was due to the mild to moderate clotting factor deficiency. It is interesting though that two cerebral aneurysms were detected in case 1. Taken together with the potentially subarachnoid hemorrhages in case 3, this may indicate that patients with this disorder might have an increased risk for cerebral aneurysms. By contrast, in PXE, cerebrovascular complications are mostly ischemic (stroke).

In order to elucidate the molecular pathogenesis of this phenotype, we initially focused on the *VKORC1* gene, as it is also located on chromosome 16p, albeit 15 Mb away from *ABCC6*. However, sequencing of the whole coding region did not reveal any causal mutation. In contrast, analysis of the second gene involved in the clotting disorder, *GGCX*, has revealed seven different mutations in six patients. Of these, p.Q374X, p.W493S, p.R476C, and p.R476H are located in the propeptide binding site of the γ-carboxylase, important for binding of the substrates. This site has been mapped to amino acids 50–225, 349–500, and 425–513 (Yamada *et al.*, 1995; Wu *et al.*, 1997; Lin *et al.*, 2002). Thus, they could result in a shorter residence time of the substrate on the γ-carboxylase with formation of poorly carboxylated, less active proteins (Mutucumarana *et al.*, 2000).

Site-directed mutagenesis demonstrated that regions around residues 234, 406, and 503 partly define the propeptide binding sites (Sugiura *et al.*, 1996). The p.F299S, p.G537Y, and p.G558S mutations may therefore also influence the affinity of the γ–carboxylase for its substrates. Further bio- and immunohistochemical analysis of the

functional effects of these mutations is currently ongoing. It is worthwhile mentioning that the vitamin K-dependent enzymes responsible for carboxylation of clotting factors are also involved in modulating bone mineralization and in preventing calcium precipitation in soft tissues (Price and Williamson, 1985; Price *et al.*, 1998). Our molecular results are remarkable in that mutations in this phenotype seem to occur typically in exons 8, 10, and 12. Mutations in these exons have not been described so far in patients with the hereditary coagulation disorder without the cutaneous phenotype. It is our hypothesis that these exons are of critical importance in domains that are essential in the activating role of the GGCX enzyme in the coagulation cascade, but also for the activation of other gla-proteins such as, for example, matrix gla protein or osteocalcin. The latter are known inhibitors of calcification. Hence, a decreased or absent activation of such proteins can explain the calcification and subsequent fragmentation of the elastic fibers. The severity of the skin lesions might be due to the additive effect of decreased or absent activity of multiple calcification inhibitors.

A second theoretical possibility would be digenic inheritance, in which a second, as yet unknown gene, is responsible for the cutaneous phenotype. Linkage analysis was possible in only one family without known mutations; unfortunately, we were unable to obtain DNA from a sib of the proband, essential for the interpretation of the results.

Furthermore, apart from gene defects, the correct carboxylation of clotting factors and of modulators of mineralization in bone and soft tissues may depend on post-translational maturation of enzymes (Wajih *et al.*, 2004) as well as on cell metabolic alterations affecting, for instance, the structural organization of membranes of the endoplasmic reticulum where carboxylating enzymes are located (Wallin *et al.*, 1999). Therefore, the problem is rather complex and the involvement of vitamin K-dependent processes in mineralization of connective tissues and of elastic fibers in particular is under investigation.

At present 10 cases with this peculiar phenotype have been reported (Table 4). In the literature, the fundus examination has always been considered normal. However, owing to the very mild aspect of the retinopathy, it is possible that small AS were missed. The fact that visual acuity has always been reported as normal is yet another indicator of the rather mild nature of the ocular features.

Bleeding tendency was very variable, as can be expected from the hereditary form of VKCFD.

In conclusion, as (i) the fundus findings and the elastic fiber mineralization in the skin of patients exclude any known form of cutis laxa, and (ii) the distinct clinical manifestations and severity of cutaneous and retinal symptoms, as well as the slightly different ultrastructural features and the absence of *ABCC6* mutations in patients seem to exclude PXE, data support the hypothesis that these patients suffer from a new disorder. The molecular background of this peculiar phenotype, and more specifically the role of the *GGCX* gene, is currently under investigation.

MATERIALS AND METHODS

Patients

The patients were clinically examined (skin evaluation, fundoscopy, and cardiovascular work-up) at the Ghent University Hospital (Belgium) (case 1), l'Hôpital Porte Madeleine, Orléans (France) (cases 2 and 3), the University of Modena and Reggio Emilia, Modena (Italy) (case 4). Patients 5 and 6 who reside in the United States were not personally evaluated by the authors, but clinical data were obtained from their medical specialists. The first four patients had a full thickness skin biopsy taken in a skin lesion suggestive for PXE. Biopsies were evaluated with light microscopy using hematoxylin and eosin, van Giesson (elastin), and Von Kossa (calcium) stains.

For electron microscopy, skin biopsy fragments were immediately fixed in 3% glutaraldehyde in Tyrode's saline pH 7.2 for 2–4 hours at room temperature, washed in saline, post-fixed in 1% osmium tetroxide in the same buffer for 1 hour, dehydrated in ethanol and propylene oxide, and embedded in spurr resin. Semithin sections were stained with 1% toluidine blue and observed by light microscopy. Thin sections were stained with 1% uranyl acetate in 50% ethanol and lead citrate and observed in a Jeol EM1200 electron microscope. Skin biopsy fragments from patients 2 and 3 were first fixed in formalin and embedded in paraffin and then rescued and embedded in spurr resin.

Coagulation assays were performed on citrated blood samples. The activities of factors II, VII, IX, and X were measured in one-stage clotting assays using Diagnostica Stago (Asnières sur Seine, France) reagents. The prothrombin time was performed using the STA-Neoplastin CI plus kit (Diagnostica Stago, France).

Informed consent was obtained from all patients and the Declaration of Helsinki Principles was followed. This study was approved by the Ethical Committee of the Ghent University Hospital.

Molecular analysis

Molecular analysis of the *ABCC6* gene was performed in all six patients and one additional, already reported, patient (Le Corvaisier-Pieto *et al.*, 1996), using PCR primers described by Wang *et al.* (2001). In order to distinguish between *ABCC6* and its two pseudogenes a long-range PCR was performed of exons 1 through 10. The following primers were used: forward primer: 5′-ATA CTC AGT ATC AGC CAG GAT GTT-3′ and reverse primer: 5′-GGG ACT CCG TTC AAA TCC CG-3′. Subsequently, PCR reactions for the separate exons were performed on the long-range amplicon. For the detection of the deletion of exons 23–29, the primers described by le Saux *et al.* (2001) were used.

The *VKORC1* coding region was analyzed using following primers: exon 1: forward primer 5′-CTC CGT GGC TGG TTT TCT C-3′and reverse primer 5′-CCG ATC CCA GAC TCC AGA AT-3′; exon 2: forward primer 5′-ATG GGA GGT CGG GGA ACA-3′ and reverse primer 5′-TGA GCA GCT AGC TGG CTG-3′; exon 3: forward primer 5′-TCT GCC CTG GAG CCT CTT-3′ and reverse primer 5′-CAC ATC TAG GGC CTT CTA G-3′.

The *GGCX* coding region was analyzed using primers and PCR conditions described by Oldenburg *et al.* (2000).

The whole coding region and intron/exon boundaries of *ABCC6*, *VKORC1*, and *GGCX* were analyzed with direct sequencing using an Applied Biosystems 3100 Sequencer, with ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Lennik, Belgium).

Nucleotide numbers are derived from cDNA *GGCX* sequences (GenBank accession no. BC013979). For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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Publication 8

Low serum vitamin K in PXE patients results in defective carboxylation of mineralization inhibitors similar to the consequences of *GGCX* mutations in the PXE-like syndrome.

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In this paper, immunohistochemical and ELISA experiments were applied on skin tissues, serum and plasma samples of PXE-like and PXE patients to determine the pattern of active (carboxylated) and inactive (uncarboxylated) Gla-proteins and associated inhibitors of calcification. Further, the vitamin K (VK) serum concentration was measured in PXE-like and PXE samples using HPLC, to evaluate the VK status.

Significant disturbances in mineralization inhibitor activation are demonstrated in the circulation and the dermis of PXE-like syndrome patients, with an accumulation of uncarboxylated matrix gla protein (ucMGP) and osteocalcin (ucOC). Serum levels of VK were normal in these patients. In PXE patients, we found similar, although not identical results for the Gla-proteins in the circulation and local dermal tissue. However, the VK serum concentration in PXE patients was significantly decreased compared to controls.

This study confirms the pathomechanistic hypothesis formulated in publication 7 on the PXE-like syndrome and allows us to conclude that ectopic mineralization in the PXE-like syndrome and in PXE results from a deficient protein carboxylation of VK-dependent inhibitors of calcification. While in PXE-like patients this is due to mutations in the *GGCX* gene, a deficiency of the carboxylation co-factor VK is at the basis of the decreased activity of calcification inhibitors in PXE.

Low serum vitamin K in PXE patients results in defective carboxylation of mineralization inhibitors similar to the consequences of *GGCX* mutations in the PXE-like syndrome

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Abstract

Soft-tissue mineralization is a tightly regulated process relying on the activity of systemic and tissue-specific inhibitors and promotors of calcium precipitation. Many of these, such as matrix gla protein (MGP) and osteocalcin (OC), need to undergo carboxylation to become active. This posttranslational modification is catalyzed by the gammaglutamyl carboxylase GGCX and requires vitamin K (VK) as an essential co-factor. Recently, we described a novel phenotype characterized by ectopic mineralization of the elastic fibers resulting from mutations in *GGCX*. Because of the resemblance with pseudoxanthoma elasticum (PXE), a prototype disorder of elastic fiber mineralization, it was coined the PXE-like syndrome. As mutations in *GGCX* negatively affect protein carboxylation, it is likely that inactive inhibitors of calcification contribute to ectopic mineralization in PXE-like syndrome. Because of the remarkable resemblance with PXE, we performed a comparative study of various forms of VK-dependent proteins in serum, plasma (using ELISA) and dermal tissues (using immunohistochemistry) of PXE-like and PXE patients using innovative, conformation-specific antibodies. Furthermore, we measured VK serum concentrations (using HPLC) in PXE-like and PXE samples to evaluate the VK status.

In PXE-like patients we noted an accumulation of uncarboxylated Gla-proteins, MGP and OC, in plasma, serum and in the dermis. Serum levels of VK were normal in these patients. In PXE patients, we found similar, although not identical results for the Gla-proteins in the circulation and local dermal tissue. However, the VK serum concentration in PXE patients was significantly decreased compared to controls.

Our findings allow us to conclude that ectopic mineralization in the PXE-like syndrome and in PXE results from a deficient protein carboxylation of VK-dependent inhibitors of calcification. While in PXE-like patients this is due to mutations in the *GGCX* gene, a deficiency of the carboxylation co-factor VK is at the basis of the decreased activity of calcification inhibitors in PXE.

Introduction

Soft-tissue calcification is a highly regulated process relying on both systemic and tissue-specific balances of inhibitors and promotors of calcium precipitation. Fetuin-A (α2-Schmid-Herremans glycoprotein), a member of the cystatin family of protease inhibitors produced predominantly by the liver, is considered an important circulating inhibitory factor of soft tissue mineralization (1). Among local regulators, matrix gla protein (MGP), osteopontin (OPN) and bone morphogenetic protein-2 (BMP-2) are associated with tissue mineralization *in vivo* (2,3,4). Proteins primarily involved in the regulation of bone mineralization, such as osteocalcin (OC) or osteonectin (ON), have also been linked to local extraosseous regulation of calcium homeostasis (5).

Among all, MGP is considered the key physiological inhibitor of soft tissue calcification, acting as a direct inhibitor of calcium crystal formation as MGP deficient mice suffer from spontaneous and ultimately fatal calcification of arteries and cartilage (6). Together with OC and the clotting factors II, VII, IX and X, MGP needs to undergo carboxylation of glutamate (Glu) into gamma-carboxyglutamate (Gla) residues to become active. Carboxylation catalyzed by the endoplasmic gammaglutamyl carboxylase (GGCX) to form Gla-residues in all these proteins requires vitamin K (VK) as a co-factor, hence the terms “VK-dependent" or "Gla"-proteins (7). MGP, unique among the Gla-proteins, also has the tandemly repeated Ser-X-Glu sequence at the N-terminus end, which is a recognition motif for serine phosphorylation. Phosphorylation is thought to be carried out by the Golgi casein kinase (8,9). It has been previously suggested that - next to being carboxylated - MGP must be fully phosphorylated at each target serine residue to have its optimal inhibitory activity on soft tissue calcification (9).

Besides the gamma-carboxylase, the metabolic VK-cycle also involves the reductase enzyme VKORC1. Mutations in the *GGCX* or *VKORC1* genes are associated with a hereditary deficiency of the VK-dependent clotting factors as well as a clinically relevant dosing dependency of anti-coagulants [(10,11). Besides these enzymatic defects, a deficiency of VK has been described in association with coagulation, bone (osteoporosis, osteoarthritis) and vascular (atherosclerosis) disorders resulting from insufficient carboxylation of Gla-proteins (12,13).

We recently described an additional phenotype resulting from sequence changes in the *GGCX* gene (chrom. 2p12; OMIM#137167). This novel autosomal recessive disorder was characterized by a generalized deficiency of the VK-dependent clotting factors as well as mineralization and fragmentation of elastic fibers leading to thickened, inelastic skin and limited retinopathy (14). This disease was coined the “PXE-like syndrome” (OMIM#610842) because of its remarkable clinical and histological resemblance with pseudoxanthoma elasticum (PXE, OMIM#264800), a prototype disorder of elastic fiber mineralization (15,16). PXE is caused by mutations in the *ABCC6* gene (chrom. 16p13.1; OMIM#603234), coding for an ATP-dependent transmembrane transporter, which is most abundant in liver and kidney (17,18). Neither the substrate of ABCC6 nor its relation to ectopic mineralization or elastic fiber changes observed in PXE is presently known.

As *GGCX* mutations in the PXE-like syndrome negatively affect protein carboxylation, we and others hypothesized that elastic fiber mineralization results from deficient VK-dependent inhibitors of calcification, such as MGP and OC. Recently reported findings already point towards a role of MGP in classic PXE, but neither the involvement of other Gla-proteins nor the mechanism for such involvement have been studied yet (19-22). Because of the remarkable phenotypical similarities of the PXE-like syndrome and PXE and the absence in PXE of *GGCX* or

VKORC1 mutations – which both have the potential of disrupting the VK-cycle -, we wondered whether the VK-cycle could also be disturbed by another mechanism in PXE. More precisely, we hypothesized that disturbances in Gla-proteins in PXE patients could be linked to VK, the cofactor in the VK-cycle and an essential mediator of Gla-protein carboxylation.
We aimed to evaluate these hypotheses by studying the various forms of Gla-proteins in serum and tissues of PXE-like syndrome and PXE patients by using innovative antibodies. The latter were designed to specifically measure and differentiate between carboxylated and uncarboxylated forms of the proteins in immunohistochemistry (IHC) as well as in ELISA assays. Furthermore, we evaluated the VK status in PXE-like and PXE patients by HPLC measurement of VK serum concentrations.

Materials and methods

Patients

PXE and PXE-like patients were clinically evaluated in the Department of Genetics of the Ghent University Hospital (Belgium), the Department of Dermatology of the Orleans Hospital (France), and the Department of Biomedical Sciences, University of Modena and Reggio Emilia (Italy). Informed consent was obtained from all patients and the Declaration of Helsinki protocols were followed. This study was approved by the Ethical Committee of the Ghent University Hospital.

Biochemical measurements

Serum was prepared by incubating the samples for 20 minutes at RT and subsequent centrifugation (10 min, 3000xg); plasma was prepared in citrate tubes. All samples were frozen within 2h after blood collection.

Mineralization inhibitor levels were quantified in serum and citrate plasma of 4 PXE-like and 16 PXE patients using the ELISA technique. For measurement of the total fraction of uncarboxylated MGP (ucMGP), a competitive ELISA assay was applied (23,24).

Two additional sandwich ELISAs were developed at VitaK BV, Maastricht, The Netherlands, to determine the respective plasma levels of desphospho carboxylated (dp-cMGP) or desphospho uncarboxylated (dp-ucMGP) MGP. In brief, monoclonal anti-dpMGP was coated to the microtiter plate. After blocking, either patient plasma or standards were incubated. The standard peptide was synthetic MGP, based on the non-phosphorylated 3–15aa sequence and either the non-carboxylated 35–54aa sequence or carboxylated 35-54aa sequence, linked with a hydrophilic spacer (Pepscan, Lelystad, the Netherlands). After incubation and washing, the standard or sample was detected using a biotinylated monoclonal ucMGP antibody or biotinylated cMGP antibody.

For measurements of uncarboxylated (ucOC) and carboxylated (cOC) osteocalcin we used conformation-specific sandwich ELISAs (Takara Shuzo Co Ltd., Shiga, Japan). Results were compared with serum levels obtained in a previously described sex- and age-matched control population of 55 individuals (25).

Vitamin K_1 serum concentrations were assessed in 4 PXE-like and in 30 PXE patients using an HPLC technique with post-column reduction and fluorescence detection, as previously described (26). None of the patients were taking VK supplements or coumarins at the time of measurement. Serum VK_1 levels in patients were compared with those of a sex- and age-matched reference population of 384 healthy men and women.

Immunohistochemistry

IHC was performed on skin biopsy specimens from 3 female patients with the PXE-like syndrome reported in a previous study (14), and on skin samples from 9 patients (1 male and 8 females) with a clinical, histological and molecular diagnosis of PXE. Normal age- and sex-matched skin biopsy samples from 7 individuals and lesional skin from 3 patients with solar elastosis or elastofibroma were used as controls.

Light microscopy was performed on tissues embedded in paraffin. Antibodies against cMGP, ucMGP, fetuin-A, ON, BMP-2, gas-6 and OC were provided by VitaK (Maastricht, the Netherlands) [Schurgers et al, 2005]. The mAb against OC and polyclonal antibodies against gas6, fetuin, OPN, ON and BMP-2 stained total protein (27). After deparaffinization, sections were heated in 0.2% citric acid at pH 6.0, washed with phosphate-buffered saline and incubated with the primary antibody. For the polyclonal antibodies, citric acid treatment was not added. All antibodies were diluted in blocking reagent (Roche Diagnostics, Mannheim, Germany). Negative controls were performed by omitting the primary antibody. Biotinylated sheep anti-mouse IgG (monoclonal antibodies - Amersham Biosciences, Little Chalfont, UK), swine anti-Rabbit IgG (polyclonal antibodies - Dako, Golstrup, Denmark) or anti-goat IgG (BMP-2 - Dako, Golstrup, Denmark) were used as a second antibody (1h at RT), followed by incubation with the avidin-linked alkaline phosphatase complex (Dako, Golstrup, Denmark); staining was performed by the AEC revelation kit (brown stain; Dako). Sections were counterstained with haematoxylin and mounted with cover slips. All labellings were performed and evaluated independently by OMV and LM to assess reproducibility. The intensity of the staining was qualified in each section as 'absent' (0), 'light' (+), 'moderate' (++) or 'heavy' (+++).

Electron microscopy was performed on skin biopsies which were fixed in 2.5% glutaraldehyde in Tyrode's saline pH 7.2 (16-20h at 4°C), post-fixed in 1% osmium tetroxide (Fluka AG Chem) in the same buffer for 2h at RT, dehydrated in ethanol and propylene oxide and embedded in Spurr resin (Polysciences Inc., Warrington, PA). Some specimens were rescued from formalin fixed and paraffin embedded biopsies. Ultrathin sections were collected on nickel grids and processed for immunocytochemistry as already described (28). Unspecific epitopes were neutralized by incubating sections on 0.5% bovine serum albumin in buffer. Monoclonal antibodies towards uncarboxylated and carboxylated species of MGP and towards carboxylated MGP were used in parallel in all experiments where controls and patient samples had to be compared. Immunoreactions were revealed by secondary antibodies conjugated with 10 nm gold particles (E.Y. Laboratories, San Mateo, CA). Controls were performed by omitting the primary antibody or by incubating sections with non-immune sera instead of the primary antibody. Sections were then stained with uranyl acetate and lead citrate and observed by transmission electron microscopy (Jeol, EM1200, Tokyo, Japan).

The intensity of immunostaining was evaluated by counting the number of gold particles per unit area. More than 20 different and randomly selected areas were analyzed and mean values and standard deviations were calculated. Since the reactivity of the two antibodies is different, comparison was made only between samples processed in parallel with the same antibody.

Statistical analysis

All data given are means of duplicate measurements. Error bars represent standard deviation. Differences between groups were compared using the two-tailed Student's t-test and were considered significant at $p < 0.05$.

Results

A. PXE-like syndrome patients

Biochemical measurements

Serum and plasma concentrations of OC and MGP (Figure 1). The total amount of OC was significantly higher in the circulation of patients compared to controls, being 17 ng/ml and 8,5 ng/ml, respectively. Elevated levels of circulating ucOC were demonstrated (a), whilst cOC levels were diminished (b), resulting in significantly higher ucOC/cOC ratio compared to the reference population (c). For MGP, both the sandwich and competitive ELISA assays were used. An increase of the dp-ucMGP isoform (d) with a very high uc/cMGP ratio compared to controls (f) was shown. Competitive ELISA measurement of total ucMGP revealed significantly decreased serum levels (g).

Serum concentration of vitamin K. The serum VK concentration in PXE-like patients was normal compared to a control population (0.58 ng/ml vs. 0.60 ng/ml in the reference population; $p>0.1$).

Pathology findings

Light microscopy. Labelling for cMGP and ucMGP was strongly positive in the mid-dermal elastorrhexis area (Figure 2A & B) and absent in elastorrhexis-free areas and in control samples (Figure 2E & F). Labelling for OC was weakly to moderately positive in the epidermis and elastorrhexis zone compared to controls (Figure 3A). OC was the only protein that also stained weakly positive in fibroblasts, compared to moderate staining in controls. Fetuin-A stained heavily in the area of elastorrhexis as well as subepidermally - in the upper papillary dermis (Figure 3B). Both OPN and BMP2 stained strongly in the elastorrhexis zone, compared to very weak or no staining in controls (data not shown).

Electron microscopy. In the dermis of PXE-like patients and controls, fibroblasts were almost negative for ucMGP. By contrast, fibroblasts were slightly positive for cMGP in controls and almost negative in patients (data not shown). In the extracellular space, elastic fibers were the only matrix constituent positive for both forms of MGP in PXE-like patients and in controls. The evaluation of the number of gold particles per 1 μm^2 on the apparently normal elastic fibers revealed that ucMGP was more abundant within the fibers of PXE-like patients compared to controls, the number of gold particles being 48±24 / μm^2 in patients and 27±8 / μm^2 in controls ($p<0.001$) (Figure 4A & 5B). The antibody recognizing ucMGP reacted with epitopes abundantly present in bulky calcium precipitates (data not shown) and in the core of calcified elastic fibers (Figure 5B). By contrast, the antibody recognizing cMGP primarily localized at the border of the finely mineralized areas within elastic fibers (Figure 5D).

B. PXE patients

Biochemical measurements

Serum and plasma concentrations of OC and MGP (Figure 1). The total amount of OC was slightly lower in PXE than in control sera, being 7,5 ng/ml in patients and 9,0 ng/ml in controls. No significant abnormalities were noted in levels of cOC, ucOC, dp-cMGP and dp-ucMGP, nor in their respective uc/c ratios using sandwich ELISA compared to control samples (h-m). The competitive assay for total ucMGP however showed decreased serum levels, similar to PXE-like patients (n). Evaluation of the phosphorylation status of MGP revealed a significantly increased level of phosphorylated over non-phosphorylated MGP.

Serum concentration of vitamin K. Median serum VK concentration in PXE patients was 0.12 ng/ml. In the reference population, the median concentration was 0.60 ng/ml ($p < 0.05$).

Pathology findings

Light microscopy. Findings similar to those of PXE-like elastorrhexis areas and fibroblasts were obtained for cMGP, OC, OPN and BMP2 (Figure 2C). Although ucMGP stained somewhat less intense in the elastorrhectic fibers compared to those in PXE-like syndrome, it was still significantly more prominent compared to controls (Figure 2D). Fetuin-A also stained significantly in the elastorrhexis zone while the sub-epidermal labelling was less prominent compared to PXE-like tissues.

Electron microscopy. As previously reported (20), in the dermis, control fibroblasts were slightly positive for both ucMGP and cMGP, and PXE fibroblasts were less positive for cMGP compared to controls (not shown). The immunolocalization of cMGP and ucMGP gave results almost identical to those in the dermis of patients with the PXE.like syndrome (Figure 5A & C). Irrespective of the calcification degree, elastic fibers were the only extracellular matrix component positive for both antibodies. The immunoreaction for ucMGP, the inactive form of MGP, was always more intense in PXE compared to control elastic fibers. Moreover, as already reported (20), it was confirmed that ucMGP antibodies were associated with either bulky and finely dispersed mineral precipitates inside elastic fibers (Figure 5A), whereas cMGP antibodies nicely and precisely localised at the border of the mineralised areas of calcified elastic fibers (Figure 5C).

C. Control experiments

In both PXE-like and PXE patients, Gla-proteins which are not involved in calcium homeostasis, such as gas-6, did not stain the elastorrhexic fibers (data not shown). MGP, OC and fetuin-A labellings did not yield a positive stain in solar elastosis or elastofibroma samples used as controls of skin conditions with dystrophic but non-mineralized elastic fibers (data not shown)

Discussion

The present study confirms that inactive VK-dependent Gla-proteins are present both in the circulation and locally in dermal tissue of PXE and PXE-like patients. While in PXE-like patients this is likely due to the *GGCX* mutations, PXE patients tend to present decreased serum levels of VK, the essential co-factor of the VK-cycle, necessary for gamma-carboxylase activity.

A. VK-dependent proteins in PXE-like patients

In this study we first evaluated the hypothesis that low levels of carboxylated species of MGP and OC results in elastic fiber mineralization in PXE-like patients affected by *GGCX* mutations. We noted a significant accumulation of uncarboxylated MGP in dermal elastorrhexis. Quantitative analysis at the ultrastructural level confirmed the abundance of uncarboxylated MGP and unusually low amounts of carboxylated protein compared to controls. Sandwich ELISA assays in serum and plasma of PXE-like patients revealed decreased levels of carboxylated forms of MGP and OC while the levels of immature forms, respectively uncarboxylated unphosphorylated MGP and the total fraction of uncarboxylated OC, were dramatically increased. As a result, carboxylated/uncarboxylated ratios of both MGP and OC were decreased. Indeed, all

these findings are the direct consequences of GGCX deficiency and are factors likely to favour calcium precipitation in tissues such as the skin.

Two findings need further interpretation. First, competitive ELISA experiments, specifically measuring total uncarboxylated MGP (dp-ucMGP and p-ucMGP), showed unexpectedly decreased serum levels. This may be explained by the high affinity of the phosphorylated ucMGP fraction (the most abundant form) for calcium in contrast to the unphosphorylated ucMGP. Hence the former may accumulate in the calcified tissues resulting in low serum levels. Secondly, labelling for cMGP and cOC was clearly positive in all PXE-like skin samples. Interestingly, cMGP and ucMGP localised in different areas of mineralised elastic fibers in patients. Similarly to what observed in the dermis of PXE patients (20), cMGP was at the border of mineralization, whereas ucMGP was heavily associated with mineral precipitates (Figure 4). The presence of carboxylated species in lesional skin and plasma indicates that the VK-metabolism was not completely abolished. Yet, the amount of cMGP and cOC accumulating at sites of calcium precipitation appear to be far below the critical threshold necessary to limit the calcification process. In a single pedigree displaying PXE-like features, Li et al. recently reported that dermal elastorrhexis only weakly expressed carboxylated MGP (22). In the latter paper both mutations detected in the *GGCX* gene were associated with different reduction in gamma-carboxylase activity. We did not measure the gamma-carboxylase activity in our own patients but it seems likely that they keep a significant enzyme activity, since about 30% of carboxylated MGP and OC was still present in the circulation, although not sufficient to avoid connective tissue calcification.

The intensity of OC labelling in middermal elastorrhexis suggests an additional local role for this Gla-protein, albeit more limited. Even though the lack of conformation-specific antibodies does not allow us to demonstrate that most OC in lesional skin is uncarboxylated, our finding of increased serum and plasma levels of uncarboxylated OC in PXE-like patients corroborates with this possibility.

Besides the local calcification inhibitors MGP and OC, we observed strong staining of the potent systemic inhibitor of calcification fetuin-A in PXE-like patient's dermis. This serum protein carries calcium phosphate and has been shown to play a major role in preventing pathological calcification (29-31). The extent of the protective mechanism by fetuin-A however appears to be less than that exerted by MGP, as fetuin-A deficient mice only develop minor calcified lesions compared to the extensive calcifications in *Mgp* -/- mice (30). However, fetuin-A is closely related to the extracellular transport of MGP. Price et al. have shown that cMGP, but not ucMGP, is carried by fetuin-A in plasma (8). This suggests that VK-dependent post-translational carboxylation of MGP is essential for fetuin-A binding and transport, and therefore could influence calcium deposition in the PXE-like syndrome resulting in a two hit mechanism for the promotion of calcification via MGP.

Measurement of VK in serum of PXE-like patients revealed normal concentrations. This confirms the hypothesis that in PXE-like syndrome ectopic mineralization is primarily the result of the *GGCX* mutations causing inactive Gla-proteins.

B. VK-dependent proteins in PXE patients

The identification of the PXE-like syndrome opened an interesting avenue of comparative research in PXE, because of the resemblance of both phenotypes. We therefore evaluated the involvement of MGP, OC and fetuin-A in PXE skin samples and observed similar IHC results as in PXE-like tissues. Biochemical measurements also revealed a decrease of serum and plasma total ucMGP, but - in contrast to PXE-like patients - normal concentrations of dp-ucMGP were

found. Unlike PXE-like samples, PXE samples were noted to present an increased ratio of phosphorylated over non-phosphorylated MGP suggesting a difference in MGP phosphorylation pattern to be an important discriminator between PXE and the PXE-like syndrome.

Contrary to PXE-like patients, none of the PXE patients had mutations or functionally relevant polymorphisms in the *GGCX* gene, which could explain a possible dysregulation of the VK-cycle. We therefore also evaluated serum concentrations of VK, the essential co-factor of this metabolic cycle. We observed a poor vitamin K_1 status in PXE patients with median serum concentrations much lower than these measured in the reference population (0.12 ng/ml vs 0.60 ng/ml, SD= 0.31, $p<0.05$) or in other disorders associated with Gla-protein-related abnormal mineralization such as Crohn's disease or osteoarthritis (13,32). In some patients (n=5), the VK concentration was below the detection limit, as observed in disorders with malabsorption of fat-soluble vitamins such as cystic fibrosis (33).

These low VK serum concentrations would suggest that gamma-carboxylation of OC is also negatively affected in PXE. Surprisingly, we did not detect abnormal OC levels in PXE serum samples, despite the IHC resemblance of labellings for OC with PXE-like syndrome, in which highly disturbed OC serum levels were noted. Osteoblasts, the cell type most expressing OC, have however a high level of low-density lipoprotein receptor-related protein 1 (LRP1) on their surface. LRP1 allows efficient uptake of the apoprotein E-containing chylomicron remnants that carry the bulk of the diet-derived VK. Therefore, it is plausible to assume osteoblasts to be more efficient in acquiring their share of the inadequate amounts of circulating VK in PXE patients than e.g. vascular smooth muscle cells in arteries or fibroblasts in skin (34).

The observation of such poor VK status in PXE patients supports the recently suggested hypothesis of VK as a candidate substrate of the ABCC6 transporter, which could provide a link between mutations in the *ABCC6* gene, deficient protein carboxylation and resulting mineralization of elastic fibers (35). As VK has been described as an enhancer of kinase function, increased intracellular VK concentrations could also explain the increased phosphorylation of MGP observed in PXE patients (36).

The increased expression of OPN, a mineral-binding phosphoprotein abundant in several mineralized tissues, in both PXE and PXE-like can be considered a rescue response to MGP dysfunction (37). OPN upregulation in mineralized aorta of *Mgp* -/- mice has previously been proposed to act as a secondary, inducible, calcification inhibitor which limits further mineralization (3). In *Mgp* -/- mice, OPN initiates removal of the mineral by macrophages (38). Of note, macrophages have also been found to be abundant near calcified areas in PXE dermis (39). MGP has been shown to negatively regulate BMP2, a known osteoinductive protein (40). The observed upregulation of BMP2 may likewise be due to MGP dysfunction.

By immunoelectron microscopy it has been confirmed that ucMGP is specifically associated with calcification in both disorders and that the scarce cMGP present in the dermis of patients is precisely localised at the boundary separating calcified from normal elastin so appearing to inhibit/limit mineralization. Previous IHC reports on ectopic mineralization, showing involvement of various glycoproteins such as vitronectin or bone sialoprotein were hampered by the low specificity of these findings (41). In the present study, samples of disorders with dystrophic elastic fibers but no mineralization, such as elastosis or elastofibroma, did not yield any MGP, OC or fetuin labellings, suggesting that our observations are highly specific for PXE and the PXE-like syndrome. Similarly, labelling for Gla-proteins not involved in calcium homeostasis, such as gas-6, were negative.

From our findings, we can conclude that ectopic mineralization in the PXE-like syndrome and in PXE results from a deficient carboxylation of VK-dependent inhibitors of calcification. In the PXE-like syndrome, this deficiency results directly from mutations in the gamma-carboxylase gene. In PXE, deficiency of the carboxylation co-factor VK is at the basis of the decreased activity of calcification inhibitors. In both disorders MGP has a critical place. Whether VK is indeed the substrate of ABCC6 needs further study. However, our observations are promising towards therapeutic interventions in PXE, a disorder with no treatment to date. It has been shown in other disorders with poor VK status that exogenous VK supplementation enhances the level of γ-glutamyl-carboxylation, increasing the levels of carboxylated Gla-proteins, and as such (partially) inhibiting calcium precipitation. In animal models with deficient VK-dependent calcification inhibitors, ectopic calcification could be partially rescued with high doses of VK (25). As it is possible that other inhibitory pathways of calcification, such as the Ank-pathway in the *Abcc6-/-* mice (42), or other genes are also involved in clinical manifestations of human PXE and PXE-like syndrome, the extent of the VK supplementation effect remains to be determined in a clinical setting.

Acknowledgments

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Figures

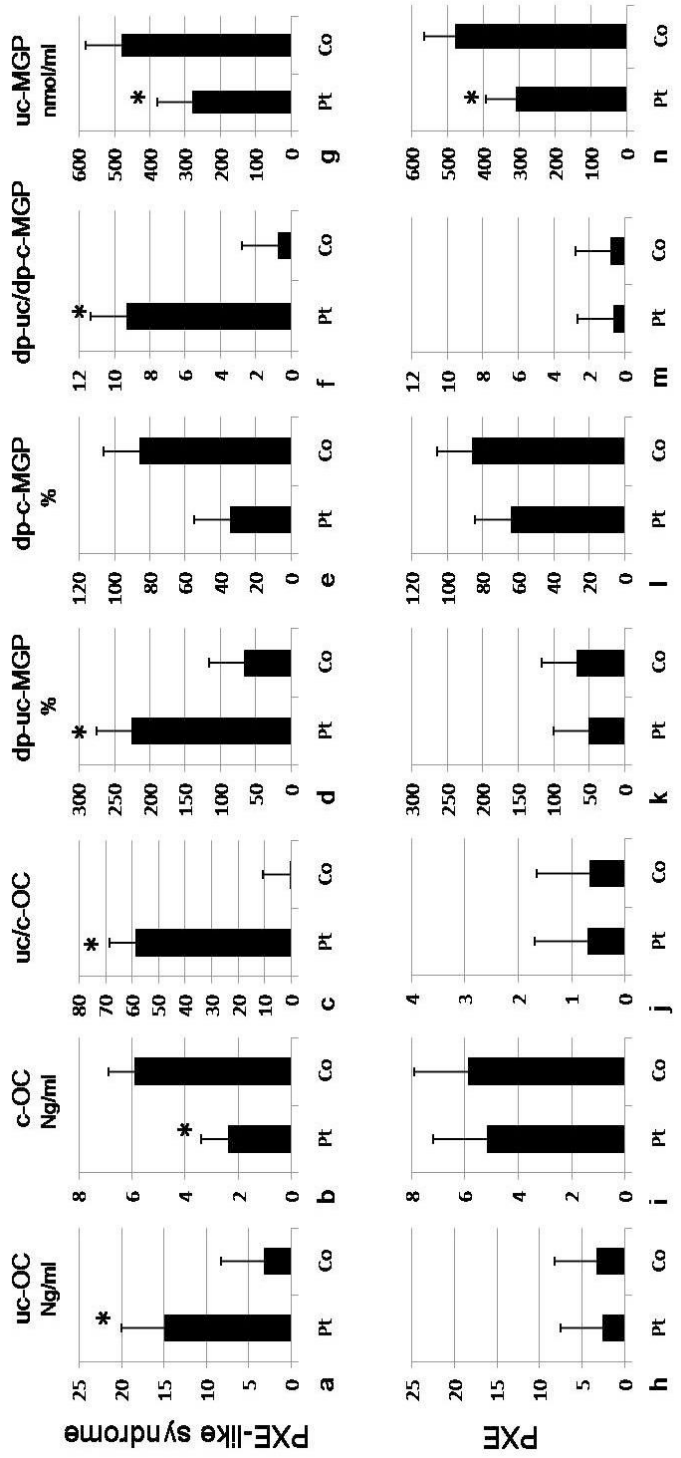

Figure 1

Sandwich ELISA and competitive ELISA (g&n) measurement results in PXE-like and PXE patients, compared to controls. uc: uncarboxylated; c: carboxylated; p: phosphorylated; dp: dephosphorylated; OC: osteocalcin; MGP: matrix gla protein; *: $p < 0.05$

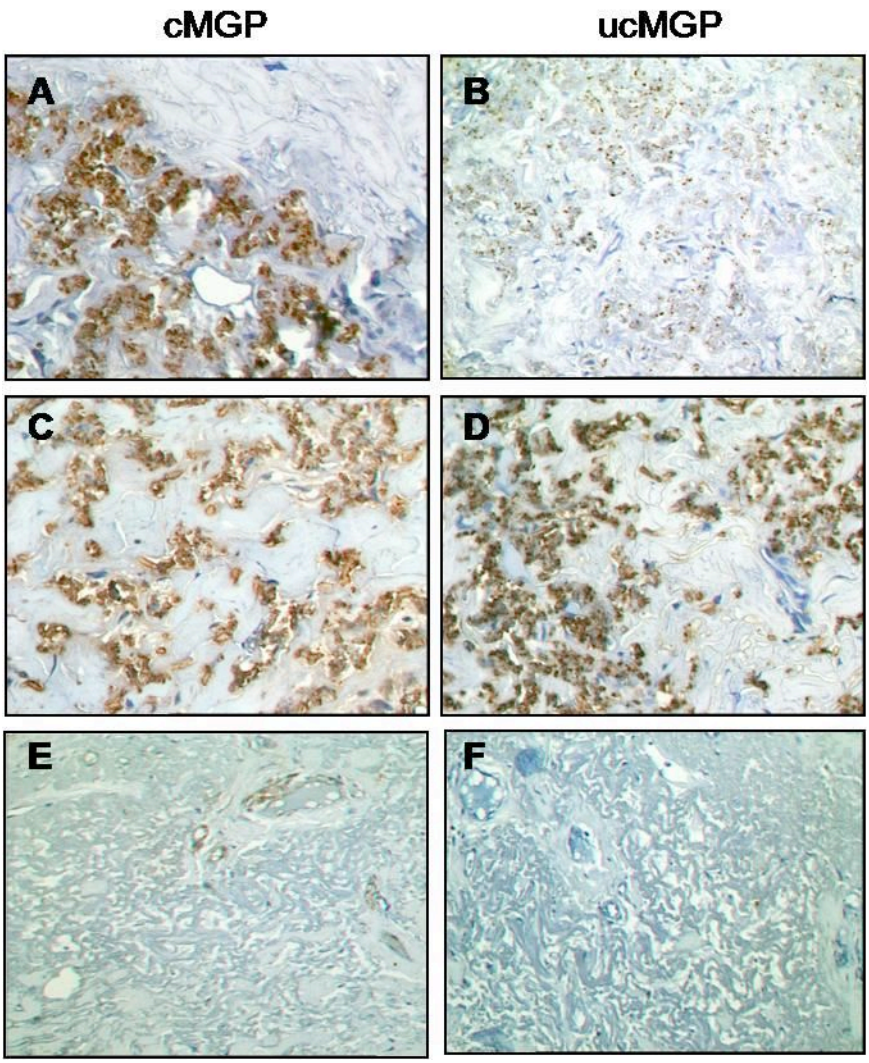

Figure 2
IHC staining results for carboxylated MGP (cMGP) and uncarboxylated MGP (ucMGP), respectively in PXE-like patients (a-b), PXE patients (c-d) and controls (e-f)

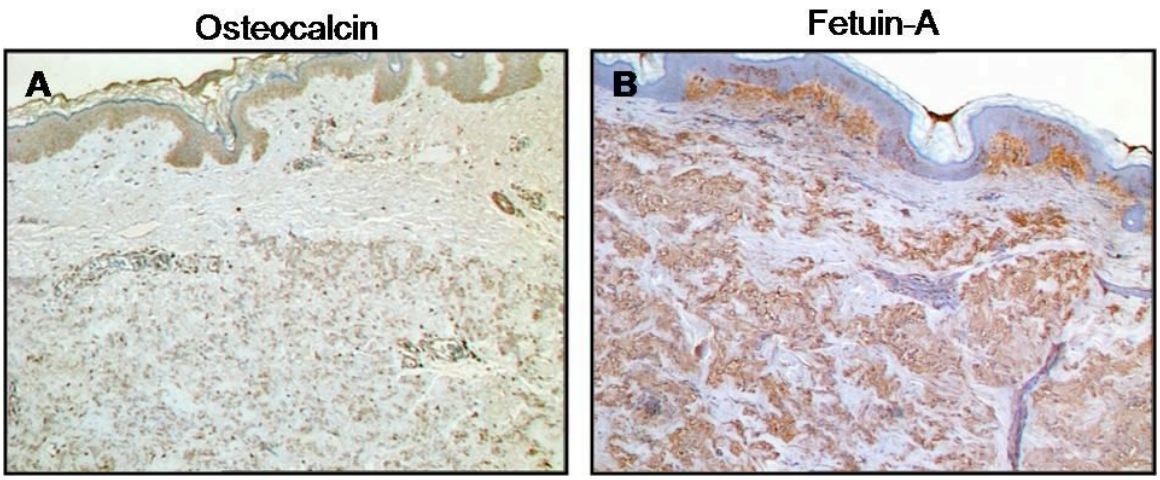

Figure 3

IHC staining for osteocalcin and fetuin-A in PXE-like patients. In PXE, similar labeling was observed

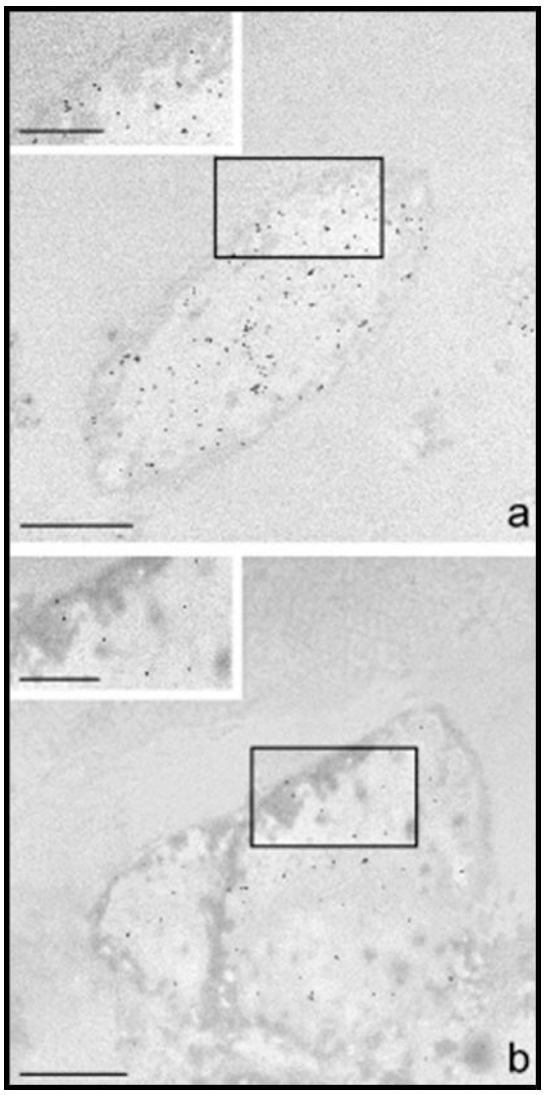

Figure 4
Transmission electron microscopy of elastic fibers in the skin of control subjects. Gold particles represent epitopes positive to anti-ucMGP (a) and anti-cMGP (b) antibodies. Bars: 1µm and 0.5µm (inserts)

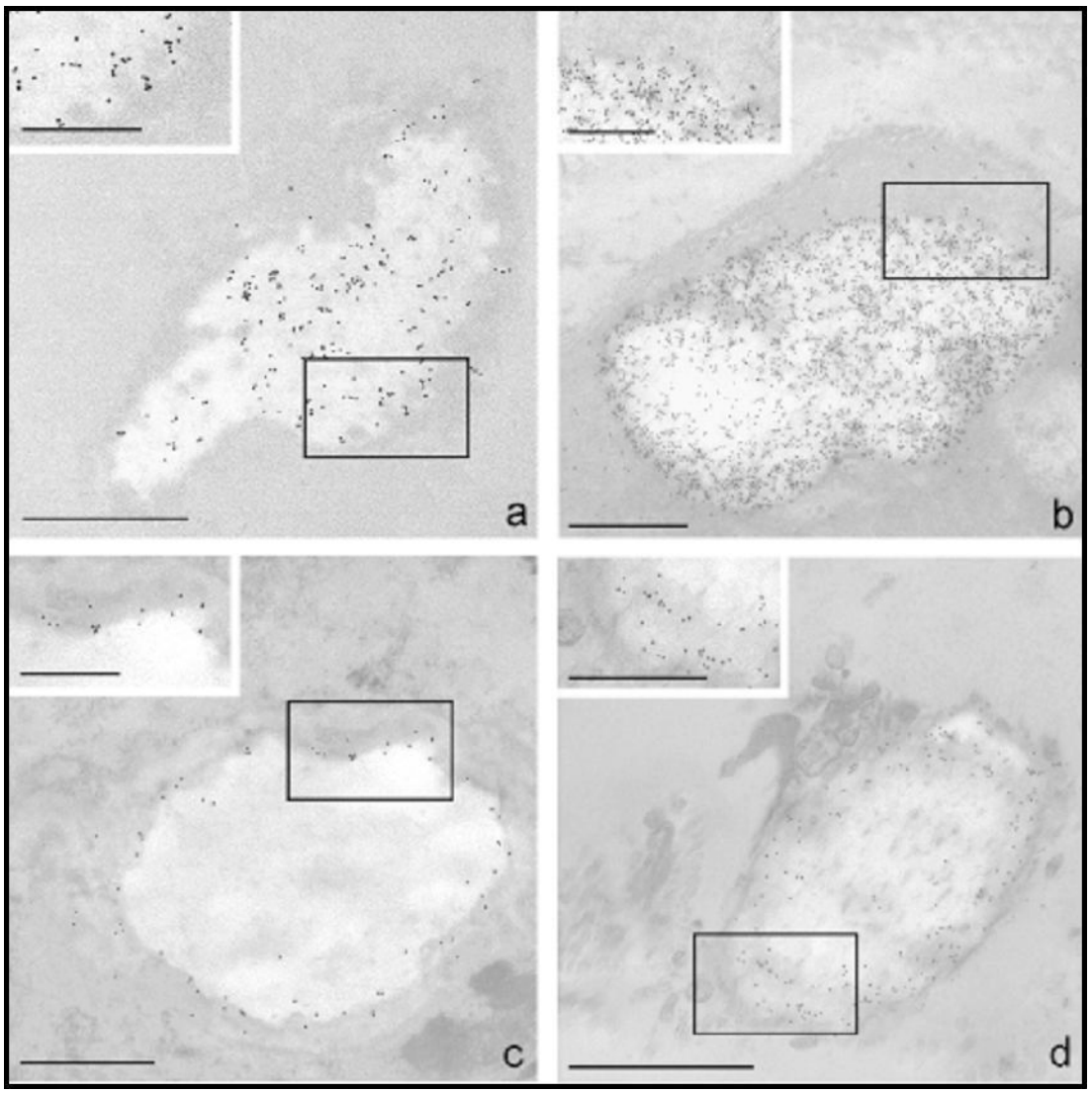

Figure 5

Transmission electron microscopy of mineralized elastic fibers in the skin of PXE (a,c) and PXE-like (b,d) patients. Gold particles represent epitopes positive to anti-ucMGP (a,b) and anti-cMGP (c,d) antibodies. Bars: 1µm and 0.5µm (inserts)

Publication 9

An atypical case of pseudoxanthoma elasticum with abdominal cutis laxa: evidence for a clinical disease spectrum

Olivier M. Vanakker, Bart P. Leroy, Leon J. Schurgers Paul J. Coucke, Anne De Paepe.

In preparation for submission to J Med Genet

In this case report, a patient is presented with a severe cutis laxa of the abdomen. Several biochemical and molecular tests, including biochemistry of the collagens and sequencing of the *fibulin-5* and *elastin* gene failed to endorse the diagnosis of a classic type of cutis laxa. Incidental ultrasonographical discovery of renal calcifications during his general work-up suggested a possible diagnosis of PXE.

A thorough clinical examination revealed a minimal yellowish reticular pattern in the anterior neck region. Skin biopsy and ophthalmological examination confirmed the diagnosis of classic PXE, albeit that on ultrastructural evaluation also calcium deposits in the periphery of elastic fibres – typical for the PXE-like syndrome - were noted. Clinically, this patient presented characteristics of both classic PXE (retinopathy, renal calcifications) and the PXE-like syndrome (cutis laxa beyond the flexural areas).

Immunohistochemical experiments and ELISA tests for various inhibitors of calcification exhibited results which were partly reminiscent of both PXE and the PXE-like syndrome. It is to be expected that this patient, and to a further extent, the PXE-like syndrome, is merely the tip of the iceberg of ectopic calcification disorders which are clinically, pathogenetically or both ways related to PXE.

An atypical case of pseudoxanthoma elasticum with abdominal cutis laxa: evidence for a clinical disease spectrum

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Abstract

In this case report, a patient is presented with a severe cutis laxa of the abdomen. Several biochemical and molecular tests, including biochemistry of the collagens and sequencing of the *fibulin-5* and *elastin* gene failed to endorse the diagnosis of a classic type of cutis laxa. Incidental ultrasonographical discovery of renal calcifications during his general work-up suggested a possible diagnosis of PXE.

A thorough clinical examination revealed a minimal yellowish reticular pattern in the anterior neck region. Skin biopsy and ophthalmological examination confirmed the diagnosis of classic PXE, albeit that on ultrastructural evaluation also calcium deposits in the periphery of elastic fibres – typical for the PXE-like syndrome - were noted. Clinically, this patient presented characteristics of both classic PXE (retinopathy, renal calcifications) and the PXE-like syndrome (cutis laxa beyond the flexural areas).

Immunohistochemical experiments and ELISA tests for various inhibitors of calcification exhibited results which were partly reminiscent of both PXE and the PXE-like syndrome. It is to be expected that this patient, and to a further extent, the PXE-like syndrome, is merely the tip of the iceberg of ectopic calcification disorders which are clinically, pathogenetically or both ways related to PXE.

Introduction

Pseudoxanthoma elasticum (PXE) is an autosomal recessive disorder, characterized by abnormalities of the skin (yellowish papules in flexural areas), eyes (peau d'orange, angioid streaks and retinal haemorrhage) and cardiovascular system (occlusive artery disease) (1,2). The histological hallmark of the disease is calcification and fragmentation of elastic fibres. PXE is caused by mutations in the *ABCC6* gene, coding for an ATP-dependent transmembrane transporter, the substrate and pathophysiological role of which remains to be elucidated (1-3).

Recently we described a novel PXE-like syndrome, in which patients develop generalized redundant skin folds beyond the flexural areas, associated with a mild retinopathy and a coagulation defect of the vitamin K (VK)-dependent clotting factors (4). Although lightmicroscopically identical to PXE, subtle differences in elastic fibre mineralization – which occurred in the periphery of the fibre rather than the fibre core – could be observed ultrastructurally. This disorder is caused by mutations in the *GGCX* gene, encoding a gamma-carboxylase. By carboxylating the glutamyl-residues of VK-dependent proteins, this enzyme is responsible for an essential posttranslational modification to make these proteins active (5). For this enzymatic reaction, vitamin K is an essential co-factor.

Both in PXE and the PXE-like syndrome, an accumulation of uncarboxylated VK-dependent calcification inhibitors – such as matrix gla protein (MGP) and osteocalcin (OC) was observed, resulting in elastic fiber mineralization (6). While in the PXE-like syndrome this was due to the *GGCX* mutations, low serum levels of VK in PXE patients were thought to be responsible for the inadequate activation of calcification inhibitors in PXE (6).

We report on a patient who presented with clinical, histological, biochemical and molecular features of both PXE and the PXE-like syndrome and as such uniquely illustrates that PXE and the PXE-like syndrome represent a spectrum of diseases.

Materials and methods

Clinical work-up of the patient was performed as described earlier (1). Skin biopsies were taken from the lateral neck and the abdominal region and were evaluated with light microscopy using Haematoxylin & Eosin, van Giesson (elastin) and Von Kossa (calcium) stains. Preparation of skin biopsy fragments for electron microscopy was done as described by Gheduzzi et al. (7). Immunohistochemistry with antibodies against cMGP, ucMGP and OC, provided by VitaK BV (Maastricht, The Netherlands) were performed as previously described (6).

Molecular analysis of the *ABCC6* and *GGCX* genes was performed as previously described (1,4). For biochemical measurements of mineralization inhibitors, serum was prepared by incubating samples for 20 minutes at room temperature and subsequent centrifigation. Plasma was prepared in citrate tubes. For measurement of the total fraction of ucMGP, a competitive ELISA assay was applied (8). Two additional sandwich ELISAs were developed at VitaK BV to determine the respective plasma levels of dp-cMGP and dp-ucMGP. For measurements of ucOC and cOC we used conformation-specific sandwich ELISAs (Takara Shuzo Co Ltd., Shiga, Japan). Vitamin K1 serum concentrations were assessed using an HPLC technique with post-column reduction and fluorescence detection, as previously described (9).

Case report and discussion

A Caucasian male, born in 1987 from non-consanguineous, healthy parents, was first seen in our department at age 18 because of suspected cutis laxa. At age 10, his mother first noticed excessive skin folds, predominantly on the abdomen. The skin was loose and reduced in elasticity with relatively gross skin folds. Progressively, this cutis laxa-like aspect became also apparent in the axillae, upper arms and elbows (Figure 1). History revealed that prior to the occurrence of these skin folds, the skin had a reddish, inflamed aspect. This inflammatory process disappeared spontaneously leaving only the residual skin laxity. At the time of consultation, a similar rash was observed on the thorax. The lower limbs and the face were never affected. Despite the history of inflammation prior to the occurrence of cutis laxa, the aspect of the skin was not suggestive for acquired, post-inflammatory cutis laxa (10). Hence, the first diagnostic tests aimed to evaluate the connective tissue by means of a biochemical analysis of the collagens and molecular analysis of the elastin (*ELN)* and fibulin-5 (*FBLN5*) genes, responsible for hereditary cutis laxa (11,12). Neither abnormalities in the collagens nor mutations in the *ELN* and *FBLN5* gene were observed.

Our suspicion was first raised towards PXE when an abdominal ultrasound during a routine check-up revealed several small renal hyperintensities. Renal microcalcifications have previously been described as a common finding in PXE patients (13). Rigorous inspection of the skin, one year after the initial examination, revealed a very mild yellowish reticular rash in the frontal neck. No other skin characteristics compatible with PXE – in particular peau d'orange or papular lesions – were noted at that time. Further work-up of this patient included an ophthalmological evaluation, revealing a best corrected visual acuity of 12/20 with correction. In fundo, peripapillar angioid streaks and peau d'orange in the midperiphery were noted in both eyes. In the left eye, angioid streaks were also present in the macula (Figure 2).

Skin biopsies were taken from the lateral neck and from the abdominal laxe skin folds. Lightmicroscopical evaluation revealed calcification and fragmentation of elastic fibres in the reticular dermis of both biopsies. No inflammatory signs were noted. On ultrastructural evaluation, the presence of fragmented elastic fibres with mineralization in the core of the fibres, typical for PXE, was confirmed (14). Also, huge fibroblasts with polymorphous nuclei and aggregates of matrix constituents, often seen in PXE, were present (14). Interestingly, also calcium deposits in the periphery of elastic fibres were observed, characteristic for the PXE-like syndrome (4) (Figure 2). Together with the abdominal cutis laxa, an atypical localisation for classic PXE but often seen in PXE-like patients, this patient was further evaluated for characteristics of PXE and the PXE-like syndrome by means of immunohistochemistry, biochemical and molecular analysis.

Immunohistochemical stainings of the affected skin, both in the neck and the abdomen of the patient, revealed marked staining of ucMGP and OC, as previously observed in both PXE and PXE-like patients (6). Interestingly, the distribution of the labelling was not confined to the middermis – as in PXE - but rather resembled the findings in PXE-like patients where ucMGP is observed throughout the whole dermis (Figure 3). Similarly, stainings for fetuin-A were of note because of the occurrence of a strong subepidermal labelling, a feature typically seen in PXE-like patients but absent in classic PXE (6).

Biochemical blood analysis, including calcium, phosphorus, kidney, liver and blood coagulation tests (PT, aPTT) were within normal limits, ruling out the co-existence of a deficiency of the vitamin K-dependent clotting factors. Measurement of circulating levels of carboxylated and uncarboxylated OC and MGP revealed elevated levels of ucOC, while cOC levels were diminished, resulting in significantly higher ucOC/cOC ratio compared to the reference population (Table 1). Such an elevated ucOC/cOC ratio was previously demonstrated in PXE-like patients (albeit even much higher than in the presented case) while PXE patients exhibit a normal ucOC/cOC ratio (6). As in PXE patients, circulating levels of dp-cMGP and dp-ucMGP were within normal limits, as well as the dp-c/dp-ucMGP ratio (6). Vitamin K circulating levels were found to be severely decreased in this patient compared to the reference population (0.15 ng/ml compared to 0.60 ng/ml in the reference population ($p<0,05$).

Gla-protein / vitamin K	Patient	PXE*	PXE-like*	Controls*
ucOC (ng/ml)	13,90	3	15	4
cOC (ng/ml)	4,35	5	2	6
ucOC/cOC	3,2	0,8	60	<0,8
dp-ucMGP (%)	44,26	50	220	65
dp-cMGP (%)	55,14	62	35	85
dp-c/dp-ucMGP	0,80	0,4	9	0,76
vitamin K (ng/ml)	0.15	0.12	0.58	0.60

Table 1

Plasma and serum measurements of circulating Gla-proteins and vitamin K. *Median values

Molecular analysis of the *ABCC6* gene revealed the patient to be compound heterozygous for the p.T941I (exon 22) and c.3507-3C>A (exon 25) mutations. Because of the clinical resemblance with the PXE-like syndrome, we also analysed the *GGCX* gene; although this analysis did did not reveal any *GGCX* mutations, the previously described p.R325Q (c.974G>A; exon 8) polymorphism was found in a heterozygous state. Surprisingly, this base pair change has been reported to have a gain-of-function effect on the gamma-carboxylase (15). Hence, we can hypothesize this variant to have a beneficial effect on gamma-carboxylation of Gla-proteins and hence the phenotype. However, because of the poor vitamin K status of our patient, it is plausible that this variant does not suffice to overrule the negative effect of vitamin K depletion on gamma-carboxylation.

In conclusion, we report on a patient who presented to the clinic with phenotypical characteristics overlapping PXE (the yellowish reticular pattern in the neck, the retinopathy and the absence of a clotting factor deficiency) and the PXE-like syndrome (the gross laxe skin folds beyond the flexural areas). The overlapping nature of this peculiar phenotype was further strenghthened by the occurrence of central (PXE) and peripheral (PXE-like) calcium deposits in the elastis fibres, by the immunohistochemical labelling revealing an accumulation of inactive inhibitors of calcification throughout the whole dermis (as in PXE-like), by the increased ratio of ucOC/cOC serum concentrations (PXE-like) in contrast to the normal dp-cMGP/dp-ucMGP ratio in serum (PXE) and finally by the poor vitamin K status as is seen in PXE patients.

At this point, molecular analysis in this patient, identifying two *ABCC6* mutations and one gain-of-function polymorphism in *GGCX* does not suffice to explain these overlapping symptoms. It is likely that mutations or functional polymorphisms in one or more genes involved in calcium homeostasis play an additional role. While it has been previously suggested that in some PXE-

like patients – presenting with extensive cutis laxa and a clotting factor deficiency – a digenic inheritance, characterized by two GGCX mutations and one heterozygous *ABCC6* mutation might occur (16), the patient presented here uniquely emphasizes that not only the molecular features but also the clinical, immunohistochemical and biochemical characteristics of PXE and the PXE-like syndrome may occur together in a single patient. These observations further expand the spectrum of PXE-like phenotypes and – in the absence of *GGCX* mutations – suggests a role for multiple genetic factors in soft tissue mineralization in general.

Acknowledgments

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Figures

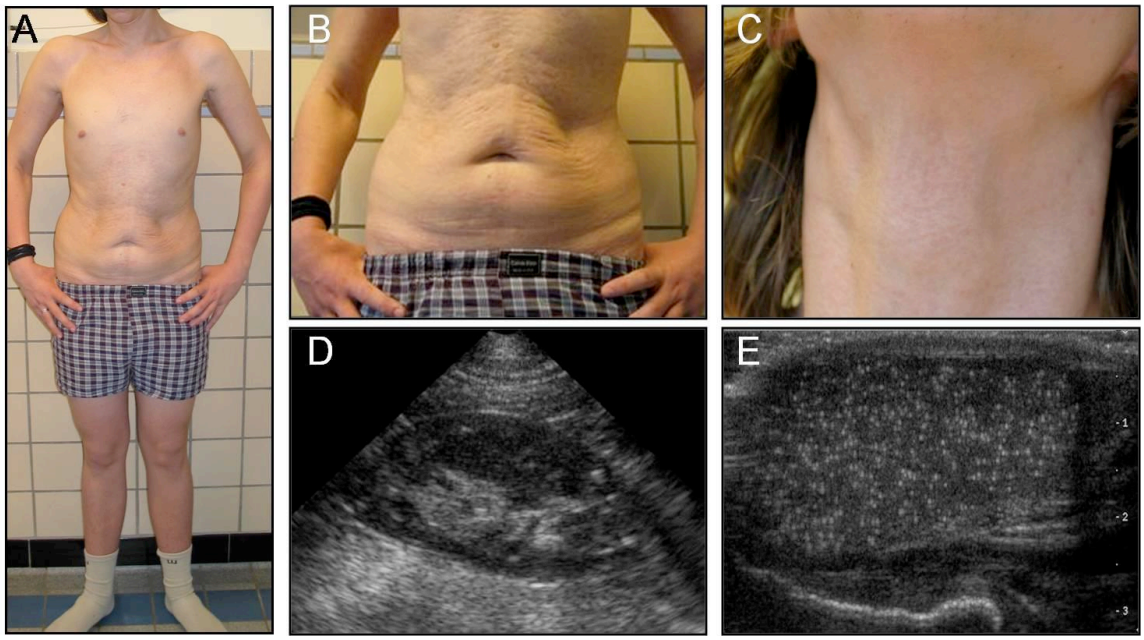

Figure 1

Phenotypic characteristics of the propositus with predominantly abdominal skin laxity (a,b), a mild yellowish reticular pattern in the frontal neck region (c), renal calcifications (d) and testicular microlithiasis (e)

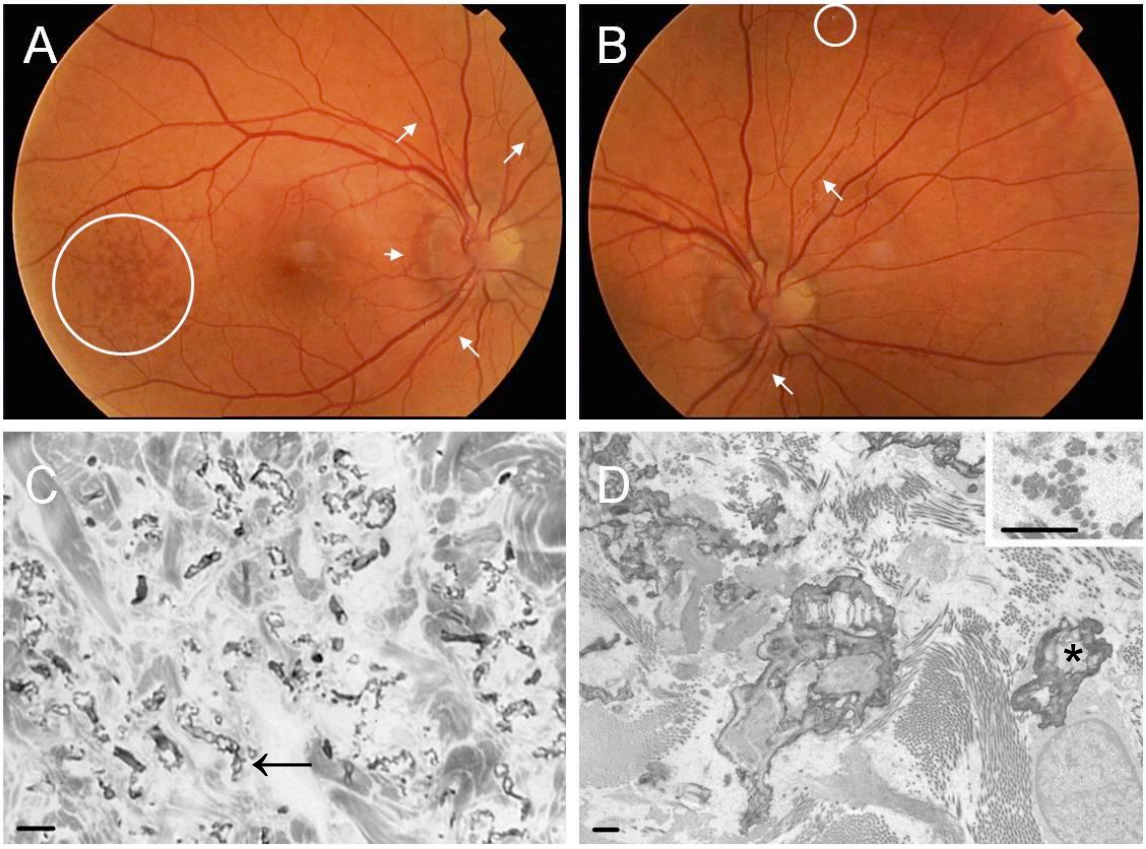

Figure 2

Ophthalmologic features with peau d'orange (a, circle), angioid streaks (b, arrowed) and 1 comet (b, circle). On ultrastructural analysis, fragmentation and calcification of the elastic fibre core (c, arrow) and periphery (d, asterisk) is observed. Bar = 1 µm

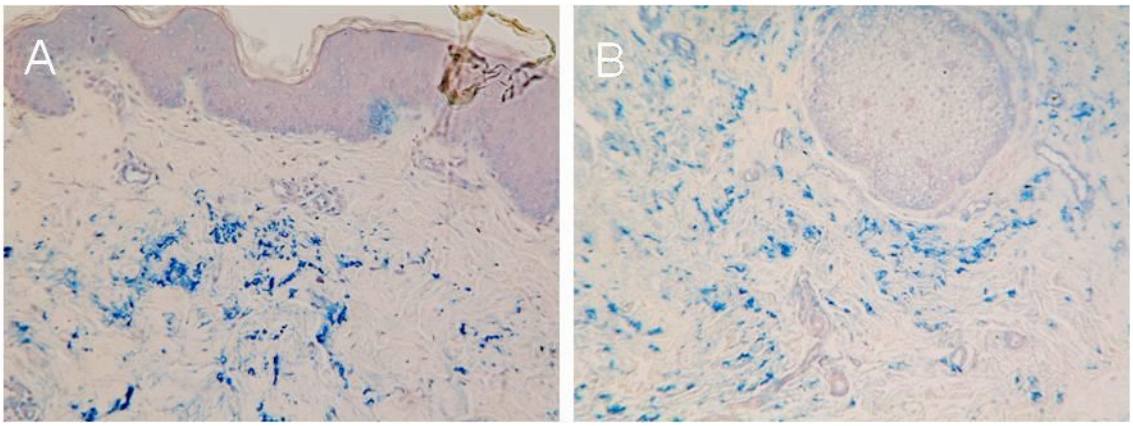

Figure 3

Immunohistochemical staining of ucMGP is significantly positive in the middermis (a) and the deeper zones of the reticular (b) dermis

Chapter 4
Discussion and future perspectives

"I hate discussions. They sometimes make you change your mind."

"Whenever people agree with me, I always feel I must be wrong."

Oscar Wilde (Thoughts & Lady Windermere's Fan)

The incentive for this thesis was to contribute to a better understanding of the mechanisms leading to PXE, as its pathogenesis remained basically unknown even more than 100 years after it was first described. To achieve this objective, we made use of **clinical studies**, **molecular techniques** as well as **basic science experiments**. From the very beginning – my early steps in the wonder world of PXE – it became clear to me that, besides the enigmatic pathogenesis, also the knowledge on **clinical aspects and natural history** of the disease lacked comprehensiveness. Indeed, disorders such as PXE – described in another century and topic of numerous publications – are easily considered as ″sufficiently characterised from a clinical perspective″. Over the years, the day to day patient care – their history, questions and concerns – has convinced me that still a large number of clinical characteristics and finesses await uncovering. As systematic unravelment of the **pathogenesis** of a disease undoubtfully requires sufficient knowledge of all aspects of clinical symptoms and signs, a number of clinical studies were undertaken, providing novel insights in known and unknown organ systems affected by PXE. Because of its relevance for diagnosis and family screening, studies were undertaken to **optimize molecular analysis** of the *ABCC6* gene and to **explore the mutation spectrum** and **genotype-phenotype correlations**. Finally, the famous adagio of translational research, "*from bedside to bench to bedside*", is put into practice by identifying a **novel, fascinating disorder**,

the etiopathogenesis of which lead to the establishment of a spectrum of PXE-like disorders and the perspective of an **innovative therapeutic approach** for such diseases.

4.1 *ABCC6* mutational spectrum and genotype-phenotype studies

4.1.1 Molecular analysis of the *ABCC6* gene

The kick-off project of this thesis comprised a thorough characterization – both clinically (see 4.2) and molecularly – of PXE patients followed at the Ghent Center for Medical Genetics. Using dHPLC and direct screening, we were able to **expand the mutational spectrum** of the *ABCC6* gene with 17 novel mutations and reported a mutation detection rate of 96% (the highest detection rate reported so far *(publication 1)*. The **high efficacy** of the molecular strategy applied in this particular study – a strategy which can be extrapolated to all future molecular studies in PXE – certainly complements the thorough clinical characterisation which is offered to each patient seen in our PXE clinic. These comprehensive **protocols** provide the patient with the most optimal health care focussed on his particular disease and gives the physician sufficient certitude on the accuracy of the clinical diagnosis. To date, more than four years after their compilation, these protocols still form the basis of a diagnostic or follow-up consultation in the Ghent PXE clinic.

The main modification in the molecular protocols over the past four years concerned the replacement of the **dHPLC** technique by direct sequencing analysis. Introduced in 2002 as an efficient, fast and reliable technique for molecular screening, experience and technological evolution turned against this method. The read-out of the chromatogram was certainly not always straight forward and required a considerable expertise (Figure 24). Although dHPLC is without question still less expensive *per se* compared to a high throughput sequencing facility, we noticed that in some patients mutations could be missed either because the chromatogram was interpreted incorrectly as normal or because it did in fact yield a normal result. As such, the speed, sample turn-over and efficacy of current partially or fully automated sequencing facilities made it a valuable substitute for dHPLC. Hence, high tech molecular genetics has evolved – in only four to five years time – from sequencing methods to screening methods to again direct sequencing of the appropriate gene.

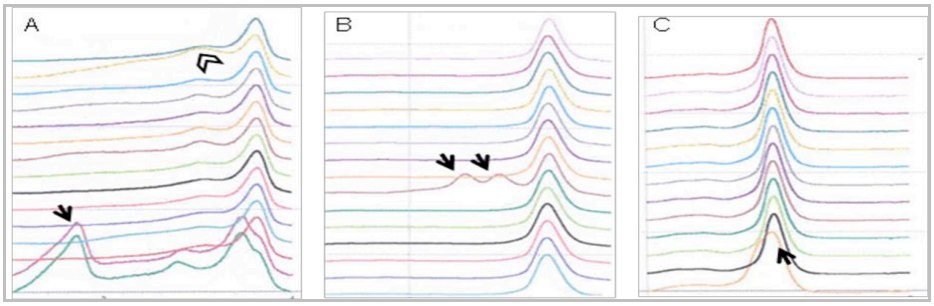

Figure 24
dHPLC chromatograms

Panel A exemplifies the p.R1141X mutation (bottom arrow), while the open arrow suggests a basepair change but actually was a normal variation. Panel B exemplifies a suggestive change, identified as the p. G755R polymorphism. Finally, panel C shows a subtle shift of the bottom curve, compatible with the c.2820-2821insC frameshift mutation.

Foremost the main shortcoming of molecular studies of the *ABCC6* gene – including our own 2007 study – is the limited number of patients in the evaluated cohort. To evade this hurdle, we worked closely together with PXE International and the South-African PXE research group in a collaborative molecular study of 270 patients. Although the study design – based on self-reporting through clinical questionnaires – made clinical characterization of the study population less extensive than our own cohort, the large number of patients enabled us to present a very **comprehensive study of the *ABCC6* mutation spectrum** (*publication 2*).

Certainly, in both studies some *ABCC6* mutations remained unidentified. This was due to the study design and limitations of the applied techniques. The recently identified **genomic aberrations** involving *ABCC6* have e.g. not been investigated. In the cohort studied by Chassaing et al., these account for 28% of unidentified alleles following direct sequencing of the gene [239]. Secondly, direct sequencing has been shown to fail in detecting **middle-sized deletions or duplications**, making further optimization of the *ABCC6* analysis strategy imperative.

The hypothesis of a second gene being involved in classic PXE seems unlikely in view of our high mutation detection rates.

A traditional topic of debate, originating from the first classification by Michael Pope, beheld the existence of one or more dominant forms of PXE [27]. Despite a growing number of authors refuting this **classification**, the occasional report describing a dominant pedigree of phenotypes designated to be classic PXE still occurred [30, 32, 33, 299, 300]. Our molecular and phenotypical studies in the Belgian PXE population as well as in a more global PXE cohort, unequivocably showed autosomal recessive inheritance to be the only modus applicable for PXE. Dominant pedigrees should first be thoroughly evaluated at the molecular level to exclude **pseudo-dominance**. In those families with a compound heterozygous genotype, all family members should be examined for both mutations known in the family. Hence, the whole ABCC6 gene should be analysed in an effort to identify two causal mutations. Indeed, it can not be excluded *a priori* that an individual designated as obligate carrier in the pedigree is in fact a patient with a mild(er) phenotype. The observation of phenotypical features in carriers, together with the lack of cutaneous signs in some patients, has rendered it difficult to exclude pseudodominance based on clinical examination. Hence, an “obligate carrier“ cannot be excluded to be a patient in a pseudodominant pedigree. Secondly, in those families where a single *ABCC6* base pair change is inherited from one generation to the next, the **causality** of this change should be critically evaluated. Indeed, it remains ever so difficult to predict the causal or functional effect of an aminoacid change, despite the standard criteria by Cotton and Scriver [301]. Finally, in the advent of increasing awareness for distinct PXE-like disorders, often with striking clinical similarities, phenotypes with undisputable autosomal dominant inheritance may represent **novel disorders related to PXE**.

4.1.2 Genotype-phenotype correlation studies

One of the most frequently asked **questions by patients,** who get the diagnosis of PXE, is what they should prepare themselves for in the future. How will their skin lesions evolve? Will these become an aesthetical burden? Will their eyesight deteriorate rapidly, condemning them to unilateral or bilateral (legal) blindness? Will the atherosclerotic or gastro-intestinal complications of the disease shorten their life span? What will be the effect of PXE on their quality of life in future years? The honest answer that every physician taking care of PXE families – when withholding from speculation – must give is also the most annoying and most difficult one – not in the least as it highlights our ignorance on some of the most vital aspects of this disease: "I do not know". The need for clear answers was a driving force behind several small genotype-phenotype correlation studies – including our own – which were not able to detect any significant association between the type or position of a given mutation and the clinical characteristics of the patient (*publication 1*). Despite the observation of **intrafamilial variability** – two or more family members with identical mutations but a completely different, sometimes opposite phenotype – a "spark of hope" remained as the studied cohorts were possibly too small to obtain significant results. This critique was however not applicable to the large study we performed together with our collaborative partners; even in a cohort of more than 200 patients, a straightforward **genotype-phenotype correlation could not be delineated** (*publication 2*).

Although these results are disappointing, they are by far not unexpected nor should they cause us to lose hope. Indeed, an extensive list of metabolic disorders exists, in which genotype-phenotype correlations were not identifiable (mitochondrial disorders, Wilson's disease, arteriopathies, skeletal disorders, storage disorders, ...) [302-305]. The high variability in clinical severity of the PXE phenotype suggests that an important role – both from the pathomechanistic and clinical point of view – is set aside for other genetic factors, such as **modifier genes** and/or **epigenetic modifications**. The modifiers identified so far though seem to have little or no clinical significance, implicating this subject as a crucial area for future research [241, 242, 306, 307].

4.2 Innovative clinical aspects of PXE

4.2.1 Soft tissue mineralization occurring in the skin and viscera

4.2.1.1 Dermatological characteristics

The skin manifestations of PXE have been an established clinical sign, leading to the diagnosis of this disorder in many patients. Indeed, as the early retinopathy is asymptomatic and cardiovascular complications usually occur in adulthood, the **yellowish papules and plaques** are often the first to be recognized by patients and physicians. During our study of the Belgian PXE population, it came to our attention that some patients had not developed typical macroscopic skin lesions at the age of diagnosis. Although some of these individuals tended to exhibit papules later in life, it was concluded that a **negative skin inspection** does not automatically rule out the possibility of PXE. Due to this particular subgroup of patients, the

discussion of sampling error – when a skin biopsy is taken blindly – arose. In the event that the biopsy is considered normal, this could merely indicate that the sample was taken adjacent to a microscopic lesion. In such cases, the molecular analysis of the *ABCC6* gene could have a genuine diagnostic purpose and replace the skin biopsy. Together with our excellent mutation uptake, this observation has significantly influenced the **diagnostic flowchart** described in publication 1.

In our efforts to study the natural history of the PXE phenotype – yet another territory which had hardly been cultivated – we noted most patients to suffer **expansion** of their skin lesions: plaques became larger or inelastic skin folds turned out to be more prominent. However, the **gradual change of skin lesion type** – from papules to plaques to inelastic dermal folds – was rarely observed. Although this is by far a general truth for all patients, it can bring reassurance for patients who are often very pre-occupied with the aesthetic consequences of their disease.

4.2.1.2 Visceral calcifications

Although occasional reports of organ calcifications – in kidney, pancreas, spleen and breasts – could be found, no systematic screening of PXE patients or healthy carriers had been performed. Following up on a 2004 case report of **testicular microlithiasis** in a fourteen-year old boy with PXE – in itself not an unusual finding in young children – we decided not to limit our study to the visceral organs but also perform ultrasounds of the testicles [172]. A remarkably high number of patients appeared to have hyperechogenic foci in one or more **abdominal organs** (*publication 3*). Such calcifications are themselves certainly not pathognomonic for PXE; several disorders are associated with metastatic mineralization (table 2); however, all of these involve abnormalities in the calcium-phosphorus balance, which are not present in PXE. As other disorders of dystrophic mineralization (scleroderma, dermatomyositis) could be clinically excluded in our patients, our observations are most likely **part of the PXE phenotype**. Indirectly, this is substantiated by the apparent concordance between visceral and ocular mineralization (comets and/or comet tails). Our recent observations in an increasing number of patients confirm these original findings. Neither in the original study cohort nor in the present patient population, significant abnormalities of kidney, liver or spleen function could be established, suggesting these lesions to be **benign**. In several patients, a progressive increase of the number of these calcified foci can be seen, indicating the need for a longer period of follow-up before drawing any definite conclusions. Publication 9 emphasizes the usefulness of an ultrasonographical screening examination of the abdomen in patients suspected to have a connective tissue and/or metabolic disease; when visceral calcifications are found to be associated with a **normal ionogram**, PXE should take a prominent place in the differential diagnosis.

Widely spread hyperechogenic foci in the testicular parenchyma, so-called **testicular microlithiasis** (TM), were observed in all male patients of the original study cohort [308-310]. The cut-off between limited and classic TM has been established on 5 calcifications. Our current clinical expertise confirms that **every male patient** followed suffers from the classical form of metastatic testicular calcification. Also Bercovitch et al. observed TM in all examined male patients [311]. Strictly speaking, TM is a histopathological diagnosis, in which small calcified lesions, so-called microliths, can be observed within the seminiferous tubules **(**Figure 25**)**. A testicular biopsy for these purposes is not justifiable in our patients, because of the risks of such a

procedure. Unfortunately, the histopathological study of Gheduzzi et al. on a deceased male PXE patient analysed nearly all tissues except… the testicles [36]. It remains sufficient to say that the ultrasonographical image – often referred to as a “snowstorm” or “heaven full of stars” – is characteristic for TM, even without histological confirmation.

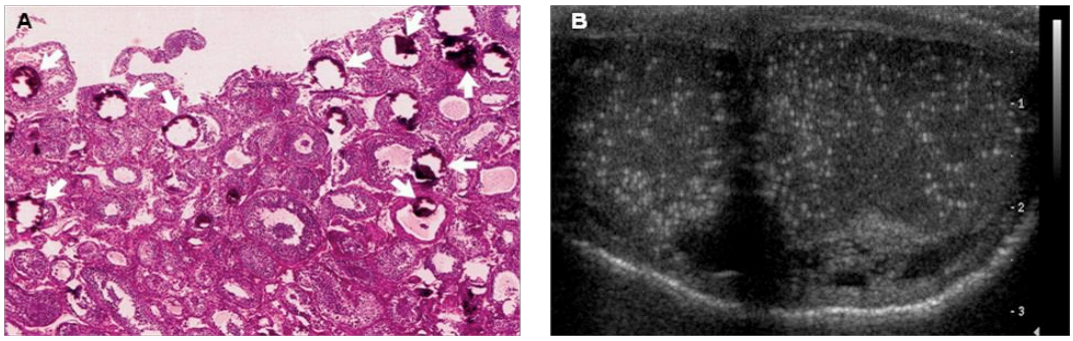

Figure 25
Histopathological image (haematoxylin-eosin stain, x8) of testicular microlithiasis (Panel A). Microliths are arrowed. Panel B demonstrates the ultrasonographical characteristics of classical testicular microlithiasis in a transverse cross-section of the testicles.
Panel A adopted from Woodward et al. [312]

Although TM is a known paediatric entity, often found in young boys and usually regressing with age, there are an increasing number of reports linking (pre-existing?) TM in adults to the **occurrence of testicular tumours** [313-353]. It must be noted that none of these reported associations were observed in a prospective study but were usually the subject of case reports or retrospective data. Hence, it remains unclear outside the field of PXE whether this presumed association is genuine or merely founded on coincidental observations. Little is known about the pathogenesis of TM or how it would contribute to the development of malignancy [308]. The fact that several other tumour types have metastatic calcifications as a secondary effect, may be an argument for a *post hoc* phenomenon. For PXE patients, the uncontrolled dystrophic calcification in various tissues could be explicatory enough for the parenchymatous mineralization of the testicles. This would imply that – even if isolated TM is linked to an increased risk for testicular cancer – the calcifications in PXE occur due to disturbance of other, probably not carcinogenic pathways. Indeed, no increase of tumours of any kind has been reported in PXE. However, until further prospective data – both on isolated TM and on testicular lesions in PXE – become available, we recommend a **yearly ultrasonographical evaluation** of the testicles and regular self-examination, as these methods have sufficiently proven their efficacy to detect testicular tumours in an early stage [354-356].

4.2.2 Ocular findings: a comet’s tale...

From the ophthalmological side, there was a need for better characterization of the **functional aspects** of the PXE retinopathy as well as **optimization** of **mild fundus lesion detection**. Our studies have indicated the ophthalmological phenotype of PXE to be composed of both anatomical and electrophysiological dysregulation of the retina. Optimal visualization of the anatomical features is achieved **combining IRI, RFI and AFI**.

4.2.2.1 Anatomical fundus injuries

Although fundus aberrations largely remained unchanged since their first description by Grönblad, the advent of novel imaging techniques made apparent that some of these lesions were underestimated or even overlooked when only white light funduscopy was applied. **Infrared, red-free and autofluorescent imaging** of the fundus are currently emerging as the standard in visualizing the damage due to ARMD and other retinal dystrophies and stand out because of their accuracy and high detection yield of limited early onset lesions [357-363]. Particularly the wet form of AMD, associated with development of abnormal neovasculature, is highly resemblant of the PXE retinopathy (Figure 26). When used to visualize PXE fundi, it was noticed that combining these novel methods lead to an earlier and more extensive visualization of both AS and peau d'orange (*publication 5*).

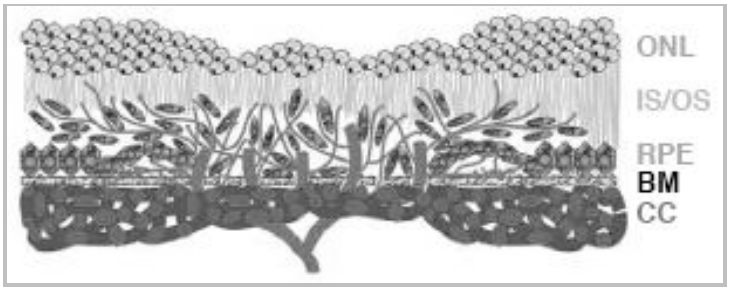

Figure 26
Pathogenesis of wet AMD. The growth of new vessels from the choriocapillaries (CC) through Bruch's membrane (BM) results in neovasculature – choroidal new vessels – both above and below the RPE. IS/OS: inner segment, respectively outer segment of the photoreceptors; ONL: outer nuclear layer.
Adopted from Tezel et al. [364]

Angioid streaks could be classified into **two types**, based on whether or not they were accompanied by surrounding RPE alterations. In the absence of such RPE changes, AFI was not able to visualize the streaks, while they were very distinct on IR imaging compared to conventional colour fundus photography. Indeed, streaks either absent or initially unnoticed on colour pictures were detected with IRI. This lead us to conclude that a **combination of AF and IR imaging** is essential for optimal visualization of the different types of AS and is as such superior to colour imaging for the early detection and follow-up of AS. Although several potential pathogenetic mechanisms could be proposed based on the autofluorescence patterns of the different types of streaks, no definite pathologic substrate could be delineated so far to explain what is actually happening in the PXE retina at the cellular level. Our data suggests decreased outer segment phagocytosis by RPE cells to result in loss of intracellular lipofuscin in the RPE cells adjacent to the streaks, while at the borders of the AS, proliferation and dysfunction of RPE cells occurs. Hu et al. hypothesized ABCC6 deficiency to lead to aberrant transport mechanisms in the retina [24]. Our imaging findings may indeed point towards an influence of ABCC6 on the photoreceptor-RPE complex. As in AMD, the immune system might play a role in attacking the RPE, due to impaired recruitment of macrophages in the subretinal space. This might explain the accumulation of lipofuscin observed with AFI, while death of RPE cells could result in atrophy and dysfunctional RPE cells in expression of various growth factors such as VEGF. On the other hand, despite the extent of damage to the RPE, it remains intriguing that, even within longstanding AS, normal areas of RPE could be observed, suggesting a focal detriment.

Peau d'orange could be observed in more detail and to a greater extent throughout the retina using IRI (*publication 5*). In contrast to its earlier described restriction to the retinal periphery, the dotted pattern could be observed as far as the macular region in some patients. Despite the invasion of this highly fragile part of the RPE, this lesion – the nature of which presently still remains uncertain – did not bring about significant visual problems. Yet, when considering peau d'orange as a first indication of PXE retinopathy, preceding AS for many years, the value of these imaging modes for early recognition of the disorder is beyond question.

Besides peau d'orange, AS and the neovascular complications, one of the most underestimated fundus characteristics of PXE are **comets or comet tails** (CT). These punched out lesions – which were found to represent spots of calcification on ocular ultrasonography (Figure 27) – have been rarely cited in literature, yet were seen closely associated with PXE [24, 119]. This could be confirmed in our studies, in which we also showed IR imaging of the fundus to be superior in detecting CT, even when they went unnoticed during standard fundus photography. As a result, in our current practice CT are used as an **indicator for ABCC6 dysregulation**, although this does not imply both *ABCC6* alleles to be mutated. Indeed, much to our surprise, we also found these distinct white dots in carriers of one *ABCC6* mutation. So far, their occurrence in other retinal dystrophies or normal fundi has not been reported. Hence, the recognition of isolated CT should bring about suspicion of at least one mutation in the *ABCC6* gene, but does not automatically imply the patient to have PXE. Upon recognition, even by chance, a thorough skin evaluation and molecular analysis of *ABCC6* is unquestionably indicated.

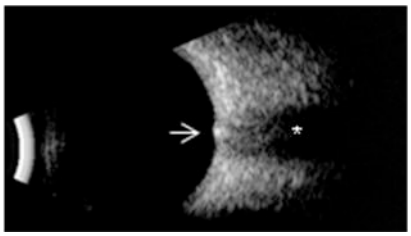

Figure 27
B-scan of a comet tail lesion, revealing a hyperechogenic spot (arrowed) with acoustic shadowing (asterisk), the typical hallmark of chalk on ultrasound.

4.2.2.2 Electrophysiological dysregulation

Some PXE patients presenting with visual complaints do not have such considerable anatomical deterioration of the RPE – even after AFI examination –to justify their decreased visual acuity. Particularly in such patients, the presence of generalized retinal dysfunction should be considered. We have described three phenotypes – **cone-rod dystrophy**, **rod-cone dystrophy** and **severe photoreceptor dystrophy** – in four young individuals with severe visual handicap due to PXE (*publication 4)*. Previous reports on retinal dysfunction are scarce and limited to older individuals with advanced disciform degeneration. The presence of such dysfunction in less deteriorated fundi may suggest it to be present in other PXE patients; this would certainly explain night vision and color discrimination problems often reported by patients. An accurate rate of occurrence of these electrophysiological problems in PXE patients remains, currently under investigation.

Because also in these individuals **no correlation with the *ABCC6* mutations** could be noted, it seems most probable that modifier gene(s) determine whether or not PXE is accompanied by such generalized retinal dysfunction. Identification of such modifiers will be the topic of future research.

Natural history study of the PXE retinopathy (*publication 1*) revealed anatomical progression in a quarter of patients, while almost half presented with **functional decline**. It was particularly the latter which – due to binocular occurrence – resulted in significant visual handicap. Altogether, these data encourage further electrophysiological studies in PXE.

4.2.3 Cardio- and cerebrovascular features

I have always had the feeling that the cardiovascular symptoms or – if you will – complications of PXE were condemned to the backstage plan after the clinically obvious and diagnostically important skin lesions and the potentially handicapping ocular signs. It is in this respect probably no coincidence that the cardiovascular phenotype was historically the last to have been described and added to the triad of clinical signs and symptoms. Yet, although undoubtfully not as frequent as the oculocutaneous signs, the cardiac and vascular symptoms are the source of **significant morbidity** and – if not detected and treated timely – **mortality**. It was and is therefore my conviction that this part of the triad deserves thorough investigation.

4.2.3.1 Ischemic stroke in PXE

Although cerebrovascular complications of PXE were seldom emphasized in the past, we observed a considerable **higher incidence of ischemic stroke** in PXE patients, usually in the fifth or sixth decade of life. This observation in a consecutive cohort corroborated several case reports [85, 139, 365-371]. These results lead to the design of an exploratory pilot study in close collaboration with the Neurology department of the Ghent University Hospital to investigate in a cohort of consecutive ischemic stroke patients whether we could either find (subtle) signs of PXE, signifying stroke to be an eventual presenting complaint as was previously shown for acute myocardial infarction [159] or whether a higher frequency of mutations in the *ABCC6* gene could be detected compared to the general population. While **no novel PXE patients** emerged from this pilot study, a **significantly higher percentage of heterozygous *ABCC6* mutations** was found in the stroke population compared to controls (*publication 6*). The idea of an ABC-transporter playing a role in the diseased brain is not novel, as several ATP-binding proteins have been implicated in drug resistance (e.g. in intractable epilepsy) as well as described in relation to ischemic stroke [372-377]. ABCB1 expression was found elevated after stroke, having a deleterious effect on neuroprotective agents [378]. Conversely, two polymorphisms in *ABCA1* – the causal gene for Tangier disease – were found to be associated with a decreased risk for ischemic stroke [379, 380].

The stroke patients in whom these heterozygous *ABCC6* mutations were found, presented with **clinical heterogeneity** – in age, additional risk factors as well as stroke type – resulting in the impossibility of delineating particular subgroups of ischemic stroke in which *ABCC6* mutations would occur more frequently. As such, the results of this pilot study do not offer guidelines for diagnostic testing of *ABCC6* in stroke. Other limitations which are characteristic for a pilot study are the small sample size and, in this particular study, the use of hotspot analysis as

a tool for molecular screening. These limitations notwithstanding, the data from this study indicate that systematic physical examination in stroke patients to uncover subclinical PXE is not useful, although it remains a laudable habit to think about the disease when confronted with **young patients lacking cardiovascular risk factors**. On the other hand, our findings are promising in implicating *ABCC6* mutations in ischemic stroke and provide a basis for future research, as detailed further.

The association of these *ABCC6* mutations – all of which were changes commonly detected in PXE families – with ischemic stroke is yet another indication that *ABCC6* heterozygosity can indeed be linked to a vascular phenotype. This is not without importance for the genetic counselling of PXE families: in obligate or potential carriers of an *ABCC6* mutation, molecular analysis should be performed to confirm the mutation, which can be considered an additional cardiovascular risk factor. Although **no direct intervention** will affect this genetic susceptibility, the presence of this additional risk factor may serve as a **catalysor** for optimizing the additional preventive cardiovascular measurements.

4.2.3.2 Cardiovascular complications

Although not a frequent vascular complication, **gastro-intestinal haemorrhages** can be severe and debilitating. During a consultation, it remains frustrating to have to bring the message to a patient that a ten percent risk subsists of suffering such bleedings – plural, as in our experience one tends to be followed by several – but not being able to pinpoint which are the determinants of such haemorrhages. As no correlation with the *ABCC6* genotype exists, one or more modifier genes are most probably involved although to date no genetic factors could be identified. Medical advice is restricted to the avoidance of systemic corticosteroids and non-steroidal anti-inflammatory drugs, known to cause erosive mucosal lesions. The increased incidence of e.g. stroke in PXE patients sometimes creates a difficult therapeutic dilemma as the daily use of antiplatelet drugs for secondary prevention after cerebrovascular disease increases the risk for haemorrhages. No ideal solution exists for such quandaries but the enteric coated form of aspirin at the lowest effective dose (80 mg) is an adequate and safe intermediate solution [381].

Other (cardio)vascular findings in our PXE patients, including the predominance of **peripheral artery disease** – in particular of the femoral arteries – and the limited incidence of mitral valve prolapse, have displaced the emphasis within the cardiovascular phenotype of PXE. The former stresses the importance of regular duplex examinations of the lower legs, which is often substituted by the easiness of carotid artery examination. Doppler examinations of both should be or remain a basic element of the routine follow-up examinations of every PXE patient.

Although high frequencies of **mitral valve prolapse** have been reported in other heritable disorders of connective tissue, such as Marfan syndrome or the Ehlers-Danlos syndrome [382], it does not appear to be an important clinical feature in PXE. Inevitably, the low incidence of this valvulopathy reflects the shift in the definition of prolapse [148]. Indeed, using echocardiography, MVP is categorized as “classic” MVP when valve leaflet thickening exceeds 5 mm, while a lesser degree of leaflet thickening is termed “non-classic” MVP. While the significance of classic MVP as a cause of serious complications (endocarditis, sudden cardiac death, CVA, ...) is beyond any doubt, the non-classic configuration could possibly be regarded as a benign variant [148, 383].

4.2.4 Heterozygous carriers of 1 *ABCC6* mutation

For disorders with autosomal recessive inheritance, heterozygous carriers of one mutation – either obligate carriers such as parents and children of the affected proband or possible carriers such as the probands sibs – are generally assumed not to be affected by the disease. However, several examples can be summarised of disorders, the carriers of which present a partial (either mild or moderate) phenotype (Table 6).

Disorder	Clinical features in heterozygous carriers	Ref.
Hemochromatosis	Porphyria cutanea tarda, acute myocardial infarction	[384]
Ataxia teleangiectasia	Increased radiosensitivity and risk of cancer	[385]
Duchenne muscular dystrophy	ERG abnormalities	[386]
Naxos disease	Whooly hair phenotype, mild RV dilatation	[387]
Werner syndrome	Genetic instability	[388]

Table 6
Examples of autosomal recessive disorders with phenotypic expression in heterozygous carriers. RV: right ventricle

In PXE, the paradigm of the unaffected carrier has long been retained, despite the observation of elastic fibre degradation in macroscopically unaffected skin of carriers [197, 198]. The results of our phenotypical studies enabled us to question this principle. We noted carriers to exhibit **soft tissue calcifications** on ultrasonography of the **abdomen**, identical to but less frequent than in PXE patients (*publication 3*). As in patients, organ function remained apparently unaffected. Furthermore, some carriers were noted to exhibit hyperechogenic foci in the **testicle**, particularly in the *capsula testis*. Only one carrier was noted to have a parenchymatous calcifcation compatible with limited testicular microlithiasis. These findings suggest testicular calcifications – and hence the theoretical tumour risk – to be much less frequent compared to PXE patients.

In addition to the abdominal and testicular mineralization, calcifications in fundo – so-called **comets and/or comet tails** – were also unveiled in carriers. Although these comets are highly specific for PXE, their presence in carriers make them not diagnostic for PXE patients but do suggest the presence of – one or two – mutations in the *ABCC6* gene. When accompanied by other features of the PXE retinopathy, the diagnostic challenge is limited. The challenge lies in individuals – particularly young persons – in whom these comets are found as an isolated lesion. These individuals are not obliged to display peau d'orange or angioid streaks yet, even when their two *ABCC6* alleles are mutated. Such an individual should be considered a PXE patient until proven otherwise and should be referred for complete work-up as stipulated above, molecular analysis of the *ABCC6* gene included. Older individuals, beyond the third decade, who feature isolated calcifications *in fundo*, are less likely to exhibit PXE but are almost certainly carrier of one *ABCC6* mutation. Also in these individuals, further work-up with *ABCC6* sequencing and clinical follow-up may be useful for themselves and their family.

For all calcifying lesions mentioned, it remains probable that they will never develop any clinical significance, although – especially for the viscera and testicles – follow-up is too limited to draw final conclusions. Comets and comet tails however do not compromise visual acuity. The importance of these calcified foci as **minor diagnostic criteria** remains – also in carriers – underrated. In contrast to the recent publication of Martin et al., in whom some carriers exhibited ophthalmological complications, we have never observed so much as ocular peau d'orange or

angioid streaks in carriers, although systematically searched for with highly sensitive detection methods (*publication 5*) [389].

Besides the diagnostic value of calcifications in carriers, our observation of a significantly increased incidence of **peripheral artery disease** (atherosclerosis) and **ischemic stroke** has important consequences for the follow-up of known PXE carriers and for genetic counselling of patients. Indeed, this observation unequivocally grants the *coup de grâce* to the principle of so-called "healthy heterozygous individuals". The phenomenon of *ABCC6* heterozygosity being associated with **increased cardiovascular risk** was further substantiated by our molecular studies in a case series of stroke patients, as discussed above.

The presence of a (limited) phenotype in heterozygotes emphasizes the need for these individuals to be **followed regularly** in a specialised clinic. Also in the follow-up of these individuals, a detailed knowledge of which clinical features a heterozygous mutation can and cannot cause, must guide the type and frequency of the examinations the person undergoes. In our PXE clinic, all carriers are submitted to a thorough clinical work-up after the first visit – considered as a *status praesens* – including duplex ultrasound of the carotids and femoral arteries, ultrasonographic examination of the abdomen, testicles and heart and an ophthalmological examination. Depending on results, a plan for clinical follow-up is made up individually. The ophthalmological evaluation is performed only once if the patient is older than 25 years, as it is our experience that features of the PXE retinopathy are always present after this age. When younger than age 25 at the first evaluation, the evaluation is repeated once beyond this age, with in the mean time annual intermediate controls.

4.3 Identification and etiopathogenetic study of a PXE related disorder: unravelling a common final pathway with PXE

4.3.1 Identification of a novel syndrome...

It was – and in retrospect still is – an indescribable vibe which went through the meeting room at a 2006 international research meeting in the Ghent Center for Medical Genetics. I had just presented a patient who for many years stood out in our cohort of PXE patients. Not only was she the only patient in whom we did not find any mutation in the *ABCC6* gene – despite our rather high mutation uptake rate –, the extreme characteristics of her phenotype on many occasions made us wonder whether this was merely the more severe end of the PXE phenotypical spectrum or if this was something else? Although she presented to our clinic in her early twenties with typical papular skin lesions in the flexural areas and at first a diagnosis of PXE was confirmed by typical lightmicroscopical findings, the natural evolution of the skin lesions was unlike any other patient we had seen. Over a period of twenty years she **developed gross, thick and leathery inelastic skin folds** which were no longer confined to flexural areas but affected the whole body except the face. In addition, she only had **minor angioid streaks** next to a typical peau d'orange and this mild fundus aspect did not change over the years. Moreover, as a result of a pre-

operative blood examination, a **deficiency of the vitamin K-dependent clotting factors** without clinical implications, was fortuitously noted.

That morning in 2006, it became clear to us that there was something more to this phenotype. Our patient was no longer a single case since at least 7 other patients apparently presented with an identical phenotype, whom were followed in 3 different genetic centers. Although all these patients initially presented typical PXE skin lesions, the subsequent loss of skin elasticity did not remain confined to the flexural areas. Although the aspect of the lesions was very similar in all seven, the age of onset was diverse, with two of them having a **cutis laxa-like phenotype** already in childhood. Light microscopic evaluation showed middermal elastorrhexis as in PXE; thorough electron microscopic evaluation did however reveal subtle differences in the type and location of elastic fibre calcification and the structure of the elastic fibre meshwork compared to PXE, supporting our hypothesis that this might be a separate genetic entity. Besides these dramatic skin changes, all had the very mild retinopathy as in our patient with peau d'orange (often regressing with age) and small angioid streaks, but lacking neovessel growth, haemorrhage and visual loss. Finally, all of these patients were found to have a deficiency of the vitamin K-dependent clotting factors – factor II, VII, IX, X – a clotting defect which in all but one patient – who suffered several hemorrhagic episodes – was a fortuitous, asymptomatic observation.

As in none of these patients *ABCC6* mutations were detected, a different gene was suspected and it was the associated coagulation defect that gave us insight into the pathway which harboured the causal gene of this novel disorder. A similar hereditary clotting disease had already been described in literature [390-392]. This autosomal recessive disorder was characterized by a clotting factor deficiency restricted to factor II, VII, IX and X – as in our patients – and was, again much like our patients, usually asymptomatic although occasionally it had been found to cause neonatal haemorrhage or menorrhagies. This genetic entity was caused by mutations in the ***VKORC1* gene** or the ***GGCX* gene**, encoding two enzymes – respectively a reductase and a gamma-carboxylase – which play a crucial role in the vitamin K-cycle [390-393]. The *VKORC1* gene is located on chromosome 16p, 15 MB from the *ABCC6* gene. This relatively large distance made a chromosomal rearrangement affecting the two genes less likely while direct sequencing did not reveal any mutation in our patients. The second candidate gene, *GGCX*, was found to be mutated in all 7 patients. Missense and nonsense mutations were found in compound heterozygous or heterozygous state. Indeed, we have so far not been able to detect both mutations in all patients. This might be due to technical limitations, although it cannot be excluded that a digenic inheritance is applicable for this disorder. Indeed, patients with the *GGCX*-linked hereditary deficiency of vitamin K-dependent clotting factors do not present any skin symptoms. Ocular symptoms have also not been described in these individuals although it must be noted that in our PXE-like patients the retinopathy remained asymptomatic and will not be detected unless it is explicitly looked for. A possible hypothesis to explain this apparent discrepancy, besides digenic inheritance, is based on the observation that all *GGCX* mutations in our patients are **confined to exons 8, 10 and 12**, which have so far not been reported mutated in the isolated clotting deficiency patients [390-392]. It could therefore be assumed that these exons encode specific domains with particular binding partners (Figure 28). Aberrant binding of one or more of these proteins or transcription factors may induce the cutaneous and/or ophthalmological symptoms. Identifying these partners may as such be valuable for the pathogenesis of PXE itself, as explained in the "future perspectives" chapter. The identification of the *GGCX* gene as the

causal genetic factor in these patients confirmed our hypothesis that this was indeed a novel disorder, which we coined **the PXE-like syndrome** (OMIM# 610842).

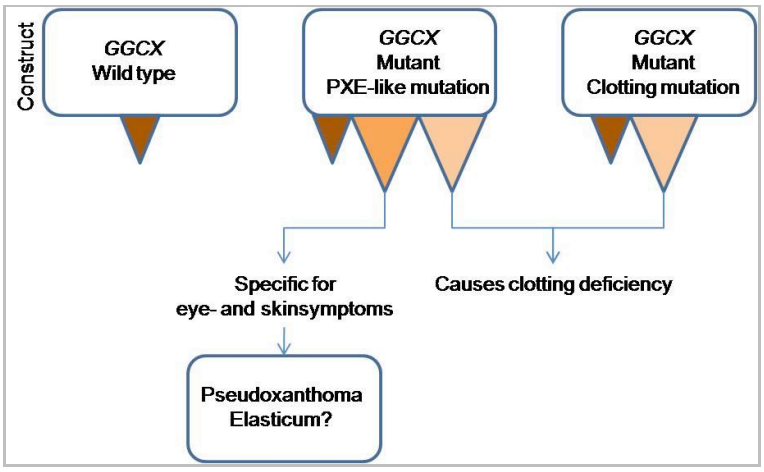

Figure 28
Hypothesis of binding factors and domains. A specific binding factor (lightly coloured large cone) causes the clotting deficiency, while a second (darker large cone) causes the skin symptoms when not being able to bind to its binding site of the GGCX protein.

4.3.2 Impact of a novel OMIM entry

The identification of this novel PXE-like disease has not been without importance for several reasons. First, it demonstrates the existence of a **spectrum of hereditary disorders resembling PXE**, although not an exact phenocopy such as the phenotype associated with haemoglobinopathies [266-271]. The concept of such a phenotypical spectrum was substantiated by the patient described in publication 9. This young man presented to the clinic several years ago at age 18 with a severe cutis laxa of the abdomen. Several biochemical and molecular tests, including biochemistry of the collagens and sequencing of the *fibulin-5* and *elastin* gene failed to endorse the diagnosis of a classic type of cutis laxa. Only the incidental ultrasonographical discovery of renal calcifications during his general work-up suggested a possible diagnosis of PXE. A thorough clinical examination with these observations in mind revealed minimal papular lesions in the anterior neck region. Skin biopsy and ophthalmological examination confirmed the diagnosis. Clinically, this patient had characteristics of both classic PXE (retinopathy, renal calcifications) and the PXE-like syndrome (cutis laxa beyond the flexural areas). Immunohistochemical experiments and ELISA tests for various inhibitors of calcification exhibited results which were partly reminiscent of both PXE and the PXE-like syndrome. Thus, also at the basic science level this patients' phenotype was situated in between classic PXE and the PXE-like syndrome. It is to be expected that this patient, and to a further extent, the PXE-like syndrome, are merely the tip of the iceberg of ectopic calcification disorders which are clinically, pathogenetically or both ways related to PXE.

Second, the characterization of the PXE-like syndrome has opened a novel avenue for **pathomechanistic research** in the field of ectopic calcification disorders, implicating local and systemic (vitamin K-dependent) mineralization inhibitors to play a critical role in this spectrum of disorders. The considerable resemblance between three diseases – PXE, PXE-like syndrome and the intermediary phenotype found in one patient – created a unique opportunity for a

comparative research project. For this, we were fortunate to collaborate with VitaK, a spin-off company of the Maastricht University (The Netherlands) who is specialized in studies involving vitamin K and vitamin K-dependent proteins. It enabled us to apply state-of-the-art antibodies and ELISA techniques in gaining further insights as to how these regulatory proteins of mineralization might be involved in one or more of these phenotypes. The results, described in publication 8, were surprising.

Since *GGCX*, the gene mutated in the PXE-like syndrome encoded an enzyme, the γ-carboxylase, known to play a critical role in the activation of **vitamin K-dependent calcification inhibitors**, clustering of the inactive conformation of these repressive proteins in soft tissues of these patients seemed a valid consequence. Providing evidence for this assumption would be a functional proof of the causality of the *GGCX* mutations we previously described. Indeed, a direct relation exists between the γ-carboxylase enzyme and the active Gla-proteins without the interference of any other enzymatic process. The **posttranslational modification** through carboxylation occurs by integrating a carboxyl (CO_2) group onto a glutamate (or glu) residue, thus conceiving gamma-carboxyglutamate (or gla-) residues [393, 394]. Although an *in vitro* method exists to verify (residual) enzymatic function of GGCX, based on the incorporation of radioactively labelled CO_2 in an artificial substrate containing glu residues, the technique is very laborious and – after some inquiries – had not been performed since many years. The use of confirmation-specific antibodies, binding specifically to the carboxylated or uncarboxylated protein, and of ELISA tests, measuring the carboxylated or uncarboxylated levels of proteins in serum, would offer a valuable and much easier alternative [395].

The **immunohistochemical labelling** of PXE-like dermal tissues revealed the presence of both carboxylated (or active) and uncarboxylated (or inactive) MGP and osteocalcin in the dermis, indicating GGCX function not to be completely abolished. It did however substantiate that carboxylation was suboptimal, thus supporting the causality of the *GGCX* mutations we ascertained. In addition to these local mineralization inhibitors, also fetuin-A – a systemic calcification inhibitory protein – was found to play a role in the pathogenesis. Although this protein is not vitamin K-dependent *per se* – it does not need to be carboxylated to become functional – it has been shown to form complexes with MGP and calcium in the serum. As such, it was possible to bring back all our findings to the vitamin K-cycle. This was also legitimate for the remarked upregulation of bone-morphogenetic protein 2 (BMP-2) and osteopontin (OPN). Indeed, MGP is a known inhibitor of BMP-2; failure of this inhibitory impulse would lead to overexpression [67]. Similarly, the *mgp-/-* mouse was shown to display OPN overexpression as a secondary effect [55]. Only recently, another posttranslational modification step became signifying an important role in MGP maturation. Indeed, the MGP protein structure is unique among vitamin K-dependent molecules due to the presence of serine residues which endure **phosphorylation** [67, 396]. This enzymatic process has been proposed to be accomplished by an enzyme called the Golgi casein kinase. It was suggested that the phosphorylation of these serine residues is a second essential step for full activation of MGP, hence the relevance of the observed increased amount of unphosphorylated MGP in the dermis of PXE-like patients [396].

It was an exciting period when we decided to perform the same labelling experiments on the dermis of classic PXE patients, our hypothesis being that, considering the clinical similarities, the pathogenetic pathways behind these disorders would be related. Indeed, the **immunohistochemical findings** were close to **identical**, in that the labelling was confined to the middermis while in PXE-like patients the whole dermis was affected. We could also observe

subtle differences in fetuin-A staining, with a repetitive positive stain subepidermally in PXE patients, which remained absent in PXE-like tissues. Although at first glance these observations seem to concur with our initial hypothesis of a common pathway, it did remain puzzling how such high similarities, if not identical, lead to two clearly different clinical entities.

We gained further insight into this intruiging ambiguity when **performing biochemical measurements using ELISA**. However, these results certainly did not simplify matters. As expected, the PXE-like serum showed perturbed ratios of active and inactive MGP and OC, the majority being the inactive form. This was a logical consequence of a mutated and less functional γ-carboxylase and was undeniably confirmed by both competitive and sandwich ELISA. In PXE patients, we noted a similar disturbance in the active/inactive ratio of MGP; however, a perfectly balanced ratio was found for OC. In addition, the competitive ELISA results for MGP failed to be confirmed by sandwich ELISA. Initially, we assumed technical error. As we obtained similar results in all consecutive patients tested, we considered the main differences between the two ELISA experiments. Besides the different technical approach, explained in chapter 2, sandwich ELISA was able to spot **uncarboxylated unphosphorylated MGP**. The competitive ELISA on the other hand was limited to detecting **uncarboxylated phosphorylated MGP** (Figure 29). These subtle changes lead us to conclude that besides γ-carboxylation, also the other posttranslational modification process – phosphorylation of serine residues – is an important determinant for which disorder was to develop.

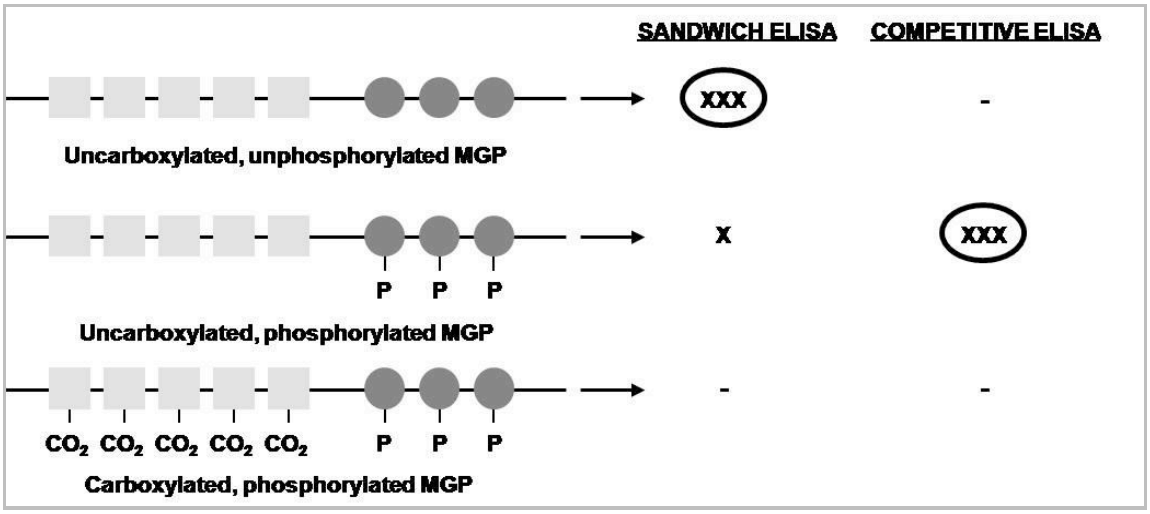

Figure 29
Specificity of the sandwich and competitive ELISA. Sandwich ELISA largely detects the uncarboxylated, unphosphorylated isoform of MGP and to a very limited extent the uncarboxylated phosphorylated isoform. Competitive ELISA only detects the uncarboxylated phosphorylated isoform of MGP.

The apparent questions remaining after these experiments were:

1/ which was or were the events leading to the accumulation of inactive VK-dependent proteins in PXE?

2/ how are these pathogenetic events related to the mutated ABCC6 transporter?

Indeed, in PXE patients no *GGCX* mutations or functionally relevant polymorphisms were found which could explain the dysregulation of VK-dependent inhibitors of calcification (Costrop et al., *in preparation*). For this reason, we looked first at the molecule serving as an essential co-factor for the carboxylation process, VK. While serum concentrations of VK in PXE-like patients were – as expected – normal, a **poor VK status** was noted in PXE, with low serum levels of VK1 compared to a reference population. Such significant VK deficiencies have been reported in a number of disorders, such as Crohn's disease and cystic fibrosis (CF) (table 7). In some of these disorders, the poor VK status has been linked to deficient Gla-proteins.

Disorder	Ref.
Crohn's disease	[397]
Cystic fibrosis	[398]
Hemorrhagic disease of the newborn	[399-402]
Osteoarthritis	[403]
Renal insufficiency with haemodialysis	[404]

Table 7
Disorders known to be caused or accompanied by poor vitamin K status

In PXE patients, average serum levels were below the average level in Crohn's disease and in some patients, unmeasurable as in CF patients. This profound VK deficiency in PXE could certainly be an adequate explanation for the accumulation of inactive Gla-proteins observed in the PXE dermis and serum. Nevertheless, some questions remain.

1/ The issue of the **normal activity of OC**. It seems unlikely that the inability to detect uncarboxylated OC would be due to technical limitations, as the method applied was identical as that in PXE-like patients. OC is primarily expressed in osteoblasts. These cells have a high level of low-density lipoprotein receptor-related protein 1 (LRP1) on their surface and LRP1 allows efficient uptake of the apoprotein E-containing chylomicron remnants that carry the bulk of the diet-derived vitamin K1. Therefore, it is plausible to assume osteoblasts to be more efficient in acquiring their share of the inadequate amounts of circulating VK in PXE patients than e.g. vascular smooth muscle cells in arteries [405]. This hypothesis however needs further study.

2/ Can disturbed Gla-protein carboxylation explain the **other phenotypical characteristics** of PXE, besides the skin lesions? Although further research is needed to confirm, literature study learns that it has been previously shown that Gla-proteins such as MGP are actively involved in **vascular calcification** related to common atherosclerosis, renal failure and diabetes vascular changes [60, 406-408]. The **ocular expression** of these proteins has not yet been studied extensively. Although MGP was shown to be present in the anterior segment of the eye, the retinal expression has not yet been investigated [409]. Gla-protein changes could also be responsible for ectopic calcification of other sites in classic PXE. We and others previously observed testicular microlithiasis to be often present in PXE patients [311, 410]; the **testicles** are known to produce an as yet uncharacterized Gla-protein [411]. In rats, OC has been shown to be expressed in male gonadal tissues, while **renal** calcifications could be due to dysfunction of Gla-proteins produced by kidney cells [412, 413]. So, it would indeed seem that all major clinical features of PXE could be explained by defects in Gla-protein function.

3/ Why are **clotting abnormalities absent** in PXE patients? It seems obvious to expect that, if a poor vitamin K status is present, clotting factors must also be affected. However, no clotting disorder – not even subclinically – has been found in PXE patients. The explanation for this apparent discrepancy lies in the observation of low VK levels in the blood; however – as there is no reason to assume malabsorption of any kind in PXE – the amount of VK1 transported via chylomicrons to the liver is normal. As clotting factors are produced and activated in the liver, enough VK1 is at hand to maintain this process. However, VK-dependent proteins involved in regulation of mineralization are local molecules, the activation of which occurs in peripheral tissues and hence is in need of peripheral VK1. The low blood levels reflect the low abundance of VK1 in these peripheral tissues.

4/ The **etiological background of the poor VK status** in PXE remains speculative. It has been established that even within the general population VK status is suboptimal. As PXE patients as a group do not explicitly differ from the general population in food habits, differences in dietary intake cannot suffice as an explanation. The most attractive hypothesis so far appears to be the idea of ABCC6 as a transporter for vitamin K or one of its precursors and metabolites [414]. While vitamin K, and in particular VK1 is efficiently taken up by the liver in chylomicrons, it needs to be secreted back into the circulation to become available in peripheral tissues (Figure 30).

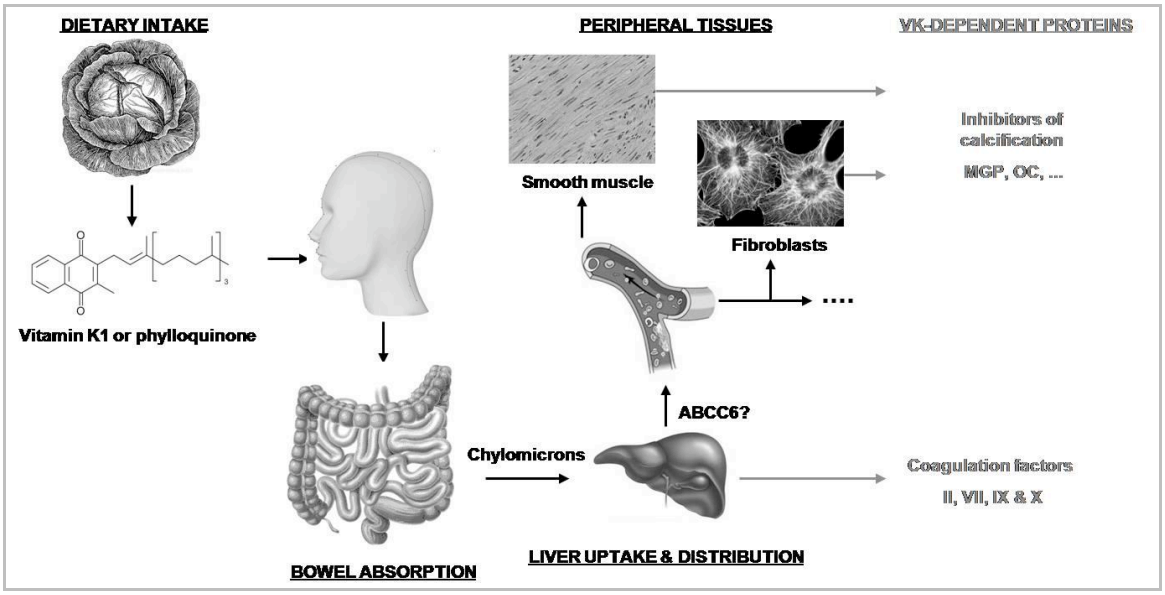

Figure 30
Vitamin K (VK) metabolism. From its dietary sources, VK1 is absorbed in the bowel and transported via chylomicrons towards the liver where it i) activates clotting factors and ii) is (partially) metabolized. VK itself and/or its metabolites are transported into the bloodstream towards peripheral tissues (smooth muscle, fibroblasts, …) where they function as a co-factor for activation of inhibitors of calcification.

How this secretion of VK by the liver is performed is not well known; so far, no VK transporters have been identified. Yet there is evidence of selectivity in secretion, suggesting the presence of such transport molecules. Additionally, the characteristics of the VK molecule – anionic properties, molecular weight – are compatible with those previously proposed for the ABCC6 substrate [414]. VK being a hydrophobic molecule

does not present an *a priori* problem, as presently no data can be found to refute an organic anion transporter having a hydrophobic substrate. Besides VK itself, the substrate of ABCC6 could also be one of the precursors or metabolites of VK. Two major compounds to consider are menaquinone-4 (MK-4) and glutathionyl-menadione (M-SG) [405]. VK1 is rapidly converted into MK-4 in the liver, which has cofactor activity in the carboxylation cycle, while M-SG – in itself not able to serve as cofactor – is turned into MK-4 in peripheral tissues [415]. Although this hypothesis seems attractive and could explain most, if not all of the PXE phenotype and pathogenesis, there are several snags to consider. In rats, M-SG is excreted in high rate into bile so that it remains to be seen whether the liver can also transport M-SG into blood [416]. One must also consider whether the rate of MK-4 formation in the liver is sufficient to provide VK to the periphery.

The hypothesis of VK as substrate for the ABCC6 transporter would also address the increased phosphorylation of MGP we observed in PXE patients, while it was not present in PXE-like patients. VK has been reported to induce activity of kinase enzymes, e.g. in cancer cell lines. The increased amount of vitamin K present intracellularly in hepatocytes due to failing transport by ABCC6, may act as such a stimulus for the Golgi casein kinase. This ER kinase is membrane-bound and could be susceptible to cytoplasmic VK.

Besides VK or one of its associated compounds, it remains possible that another cofactor of protein carboxylation serves as ABCC6 substrate. Although this would also explain the observed accumulation of uncarboxylated Gla-proteins in tissues and blood, it could not immediately explain the poor VK status in PXE patients.

Whether VK is the substrate or not, our findings – including the influence on protein carboxylation and phosphorylation – implicate for the first time one or more distinct intracellular processes – next to the observation of oxidative stress, e.g. – being involved in PXE. It suggests a complex chain of intracellular events which finally leads to ectopic mineralization of elastic fibres. They also put further in perspective the exact order in which elastorrhexis tends to occur. As said previously, it has never been unambiguously shown whether calcification preceded fragmentation of elastic fibres or *vice versa*, as both mechanisms are theoretically plausible. The model presented above suggests the fragmentation to be a secondary effect of the *primum movens*, calcification of elastic fibres. Finally, our results have taken the attempts for designing therapeutic intervention trials in PXE to another level, by implicating this vitamin and the proteins depending on its activity for their function in PXE and PXE-like disorders.

As such, my work has reached the implementation of a research adagio which I have cherished from my early clinical and research aspirations on and which remains an important drive to me until present day: from bedside to bench to bedside.

4.4 Future perspectives

After describing what lies behind, it seems imperative to take a look at the close and more distant future of PXE research. From a clinical point of view it remains important to further characterize and refine the phenotype of classic PXE. For this, several studies are currently

ongoing or being designed for the near future. Questions to be answered concern the frequency and features of electrophysiological retinal disturbances in a larger cohort of consecutive patients as well as the genetic determinants making some patients more prone to these functional aberrations than others. Cardiovascular research should endeavour into the functional consequences of cardiac and vascular elastic fibre mineralization as well as involvement of endothelial dysfunction, so far unreported in PXE but known to be a significant actor in the atherosclerotic process [417]. The findings reported in our exploratory study on ischemic stroke should be validated in other studies, enrolling larger sample sizes as well as subgroups of patients depending on age, risk factors, stroke type and ethnic background.

Reporting on natural history will also be of importance in the future. For some of the minor phenotypical features of PXE described in this thesis, such as abdominal and testicular calcifications, follow-up data are still limited. Careful prospective re-evaluation of these lesions over the next years will give us more insight into whether they are indeed rather innocent traits of PXE, useful in some cases for making the diagnosis but of no clinical significance, or whether they do influence organ function and/or cell differentiation, leading to organ dysfunction or tumorogenesis.

Although we obtained a high mutation uptake through molecular *ABCC6* analysis, it remains unsatisfying that some patients' genotype is only partially or not at all characterized. Even though – in part – this most probably is due to technical limitations (despite our expectations for molecular genetics, no detection technique has proven to be flawless), other molecular basis should be excluded. So far, the work on the *ABCC6* promotor has been limited, not in the least because of its complexity and high homology with the *ABCC6* pseudogenes. Yet this fundamental element of the gene may harbour causal mutations in some of the patients. Secondly, direct sequencing of a gene has been known to fail in detecting middle-sized deletions and duplications. However, novel techniques have been developed to study this specific type of mutation. One such method, MLPA (multiplex ligand probe amplification) is currently being used by our research group with promising results (Costrop et al. *manuscript in preparation*). Although the hypothesis of a second gene involved in PXE seems unrealistic and far-fetched to me, it must be kept in mind that distant regulatory elements in *cis* or *trans* have been reported to harbour causal mutations in other diseases; at this time, a similar molecular basis in a limited number of patients cannot be excluded. Current knowledge on transcriptional regulation is scarce and mainly comprises the identification of HNF4α (Hepatic Nuclear Factor 4 Alfa) and NF-E2 (Nuclear Factor Erythroid 2) as enhancers of the murine *Abcc6* gene promotor activity. Recently, also genes from the *PLAG* family of transcriptional factors have been put forward as regulators of ABCC6 [215]. The genes encoding these transcription factors may serve as useful candidates for further molecular testing.

Although PXE is widely known to be a disease with prominent clinical variability, this is often wrongfully interpreted as large variability in symptoms. Yet, the number of different clinical manifestations is rather limited; conversely, the severity by which patients are affected is significantly different between and within families. As we have ruled out the influence of the *ABCC6* genotype on disease severity, it is likely that modifier genes play a crucial role. Although several reports were published regarding polymorphisms in genes encoding for MGP or OPN, the true influence of these base pair variants on the PXE phenotype is always doubtful, restricted and certainly not useful in a clinical setting. The search for clinically relevant modifiers proves to be

not as forthright as one might have imagined or hoped. This has become clear through our search for modifiers making some PXE patients more prone to gastro-intestinal haemorrhage (Costrop et al. *manuscript in preparation*). Yet, for clinicians and patients, the identification of modifying variants should be continued in future years.

With the implications of vitamin K-dependent proteins in PXE and the PXE-like syndrome, the possibility of vitamin K as a potential therapeutic approach became apparent. Because clinical trials using vitamin K supplements have been done before in patients with other disorders characterized by ectopic mineralization, such as renal dialysis patients, similar clinical trials in PXE should be feasible on short term [418]. Indeed, a large multicenter trial is currently being designed to test the hypothesis that vitamin K suppletion may have a biological and – in a second phase – clinically significant effect in PXE patients.

Finally, when looking back to the implications of the identification and etiopathogenetic unravellment of the PXE-like syndrome for the field, I am convinced that we should endeavour to identify and characterize both clinically and molecularly novel phenotypes within the PXE spectrum, as they can give valuable information, not only on the pathways involved in PXE and its related disorders but on elastic fibre homeostasis in general.

Summary

"If others can see it as I have seen it, then it may be called a vision rather than a dream"

William Morris (*News from Nowhere, 1892)*

"Some states consider all visionaries to be madman"

William Blake (*Laocoön, 1826-27)*

Soft tissue calcification in the human body can be considered part of a process of continuous degeneration which we tend to designate as "aging". Being an example of technological wit and superb bio-engineering second to none, even the decay of this *corpus* can hardly be considered a random or passive event. On the contrary, calcium precipitation is regulated quite tightly by an intruiging interplay between stimulatory proteins and inhibitory factors. Thus, it has been foreseen man not to be turned into a chalk pillar in his prime years, but rather to endure a much slower process of gradual mineralization. But when this brilliant regulatory *opus* starts failing, the reign of human pathology is entered, confronting the body with ectopic mineralization disorders.

One of the archetypes of such disease is pseudoxanthoma elasticum or PXE, in which ectopic mineralization of elastic fibres causes skin, ocular and cardiovascular complications. Despite its identification more than two centuries ago, PXE has – as many genetic disorders – always been surrounded by a haze of mystery. It is the aim of this thesis to contribute to the clinical, molecular and histopathological characterization of this fascinating disease.

Through careful characterization of the PXE patient cohort followed at the Ghent Center for Medical Genetics, we were able to emphasize important clinical features, such as stroke and peripheral artery disease, as well as identifying novel phenotypical features in patients and carriers, among which were abdominal calcifications and testicular microlithiasis. Also the question of a limited or subclinical phenotype in PXE carriers was addressed and we showed them to be more prone to cardiovascular disease, next to limited ophthalmological symptoms represented by comets and comet tails.

In an exploratory pilot study among over 200 consecutive ischemic stroke patients, *ABCC6* hotspot analysis yielded a significant increase in *ABCC6* mutations compared to a healthy reference population. This signified another example of heterozygous carriers being prone to cardiovascular and/or cerebrovascular disease and introduced the *ABCC6* gene in stroke research.

In single and multi-center studies, this thesis contributed to the characterization and expansion of the *ABCC6* mutation spectrum, as well as the exclusion of genotype-phenotype correlations. The applied molecular strategy for mutation analysis of the *ABCC6* gene proved to be an efficient and cost-effective method, yielding the highest mutation detection rate so far. Also, the continuous discussion on the mode of inheritance and in particular the existence of an autosomal dominant form of PXE could be addressed constructively.

Throughout the clinical follow-up of PXE patients, we applied novel fundus imaging techniques, such as autofluorescence and infrared imaging, with substantial improvement of the

diagnostic capacities of limited or subtle lesions *in fundo*. Through collaborative efforts, the importance of electrophysiological abnormalities – subdivided in three retinopathy phenotypes – was brought to attention.

Within the span of this PhD thesis, a novel phenotype was identified and characterized both clinically and molecularly. This novel autosomal recessive disorder was coined the PXE-like syndrome, because of its resemblance with classic PXE, and was proven to be caused by mutations in the *GGCX* gene. Encoding the gamma-carboxylase, an enzyme important in the vitamin K (VK)-cycle, this observation implicated VK and proteins depending on this vitamin – among which are several inhibitors of mineralization – in the pathogenesis of the PXE-like syndrome and hence PXE.

Through various immunohistochemical and ELISA methods, VK-dependent inhibitors of calcification were shown to be inactive or defective in these syndromes, leading to ectopic mineralization in the PXE-like syndrome but also in PXE patients. These observations could be attributed to the *GGCX* mutations in the PXE-like syndrome. The observation of extremely low VK serum levels – an essential co-factor for protein carboxylation in the VK-cycle – in PXE patients explained why the VK-cycle is defective in PXE. The exact link with the impaired ABCC6 transporter remains unclear, although it is tempting to think of VK or one of its associated molecules as the substrate of ABCC6. Also, these findings hold out the prospect of VK suppletion as a treatment for PXE.

As such, the findings summarized in this thesis have elaborated the clinical and molecular knowledge of PXE and related disorders, and have opened novel avenues for further fundamental and applied research in the field of ectopic mineralization. Above all, they have benefitted patients and their family though a more efficient molecular diagnosis, a more to-the-point follow-up and the prospect of a treatment for their burdensome disease.

Samenvatting

"Dit Florentijnse patriciërsgeslacht degenereerde van generatie tot generatie en tenslotte werd kinderloosheid binnen de familie erfelijk."

J.G.A. Galeltti (*Ich sehe viele, die nicht da sind, 1987)*

Mineralisatie of verkalking van de weke delen van het menselijk lichaam kan beschouwd worden als een onderdeel van een voortdurend degeneratief proces dat we doorgaans "veroudering" noemen. Als een voorbeeld van technologisch vernuft en uitmuntende bio-engeneering zonder gelijke, kan zelfs de aftakeling van dit "*corpus humanum*" nauwelijks als een willekeurig of passief gebeuren beschouwd worden. Integendeel, de neerslag van calcium wordt zeer strikt geregeld door een intrigerend samenspel van neerslag-inducerende eiwitten en inhiberende factoren. Het is aldus zo geregeld dat we niet reeds tijdens onze jeugd veranderen in een krijtpilaar, maar dat we daarentegen een traag en geleidelijk proces van mineralisatie ondergaan. Doch wanneer dit brilliante regulerende *opus* niet meer optimaal werkt, worden we geconfronteerd met een bijzondere groep van ziektes: de ectopische mineralisatie-aandoeningen.

Eén van de archetypes van dergelijke aandoeningen is pseudoxanthoma elasticum of PXE, waarin ectopische verkalking van elastinevezels aanleiding geeft tot huid-, oog- en cardiovasculaire symptomen. Ondanks dat PXE ruim twee eeuwen geleden voor het eerst werd beschreven, blijft de ziekte – zoals dit het geval is voor vele genetische aandoeningen – omgeven door een sluier van mysterie. Het doel van deze thesis is dan ook een bijdrage te leveren tot de klinische, moleculaire en histopathologische karakterisatie van deze fascinerende aandoening.

Door zorgvuldige klinische karakterisatie van de groep van PXE patiënten gevolgd in het Gentse Centrum voor Medische Genetica, waren we in staat om het belang van bepaalde klinische kenmerken, zoals herseninfarcten en perifeer vaatlijden, te benadrukken. Daarnaast identificeerden we nieuwe fenotypische kenmerken in patiënten en heterozygote dragers, waaronder calcificaties van de buikorganen en testiculaire microlithiase. Ook het al dan niet aanwezig zijn van een beperkt of subklinisch fenotype in PXE dragers werd aangekaart en we toonden aan dat deze individuen een hoger cardiovasculair risico hebben, naast beperkte oftalmologische karakteristieken o.v.v. comets en comet tails.

In een pilootstudie in een groep van meer dan 200 patiënten met een herseninfarct werd d.m.v. *ABCC6* hotspot-analyse een significant hoger aantal heterozygote *ABCC6* mutaties terugevonden, in vergelijking met een controle groep. Dit betekent een bijkomend argument dat heterozygote dragers een hoger risico voor hart- en vaatziekten hebben; bovendien introduceert deze studie het *ABCC6* gen in stroke onderzoek.

Via lokale en multi-centerstudies draagt deze thesis ook bij tot de beschrijving en uitbreiding van het *ABCC6* mutatiespectrum, alsook het uitsluiten van relevante genotype-fenotypecorrelaties. Van de moleculaire strategie welke werd aangewend voor mutatie-analyse van het *ABCC6* gen, werd aangetoond dat ze efficiënt is, met een uitstekende kosten-baten verhouding en een mutatie-detectieratio die tot dusver de hoogst gerapporteerde is. Ook de aanhoudende discussie omtrent de overervingsmodus van PXE, in het bijzonder het al dan niet voorkomen van een autosomaal dominante vorm, werd onderzocht.

Tijdens de opvolging van PXE patiënten hebben we gebruik gemaakt van nieuwe beeldvormingstechnieken van de oogfundus, zoals fundus autofluorescentie en infrarood

beeldvorming, die een duidelijke verbetering met zich meebrachten van de diagnostische mogelijkheden bij beperkte of subtiele letsels *in fundo*. In samenwerking met Moorsfield Eye Hospital te Londen brachten we ook het belang van electrofysiologische afwijkingen van de retina – waarin drie verschillende fenotypes konden worden onderscheiden – onder de aandacht.

Tijdens dit doctoraatsonderzoek hadden we de mogelijkheid een nieuwe aandoening als aparte entiteit te identificeren en, zowel op klinisch als moleculair niveau, te karakteriseren. Deze nieuwe autosomaal recessieve ziekte werd het PXE-like syndroom genoemd, omwille van de vele gelijkenissen met klassieke PXE. We toonden aan dat de ziekte wordt veroorzaakt door mutaties in het *GGCX*-gen, dat codeert voor een gamma-carboxylase. Dit enzyme speelt een belangrijke rol in de vitamine K (VK)-cyclus. Aldus waren we in staat om VK en eiwitten die van dit vitamine afhankelijk zijn – waaronder verschillende inhibitoren van verkalking – te introduceren in de pathogenese van het PXE-like syndroom, doch ook van PXE.

Via verscheidene immunohistochemische en ELISA experimenten konden we aantonen dat verschillende VK-afhankelijke inhibitoren van calcificatie inactief of niet werkzaam waren in deze twee syndromen, hetgeen aanleiding gaf tot de ectopische verkalking. Deze bevindingen konden in het PXE-like syndroom teruggeleid worden tot de mutaties in het *GGCX* gen. In PXE werd de verklaring voor deze deficiënte eiwitten gevonden door het aantonen van zeer lage hoeveelheden VK – een essentiële co-factor voor de carboxylatie van eiwitten in de VK-cyclus – in het bloed van PXE patiënten. Hoe de gemuteerde ABCC6 transporter in relatie staat tot deze lage hoeveelheden VK is op dit ogenblik onduidelijk, hoewel het verleidelijk is te denken dat hetzij VK, hetzij één van zijn precursoren of metabolieten, door ABCC6 wordt getransporteerd. Bovendien bieden deze bevindingen het vooruitzicht van VK suppletie als behandeling voor PXE.

Zoals hierboven beschreven, hebben de resultaten van dit doctoraatsonderzoek geleid tot een uitbreiding van de klinische en moleculaire kennis over PXE en aanverwante aandoeningen. Er werden nieuwe wegen geopend voor verder fundamenteel en toegepast onderzoek binnen het gebied van ectopische mineralisatie. Boven alles zijn het de patiënten en hun familie die baat hebben bij onze resultaten, door een efficiëntere moleculaire diagnose, een to-the-point follow-up en het vooruitzicht van een behandeling voor hun belastende aandoening.

Résumé

"Le fils d'un eunuque eut mille enfants, cré tonnerre! Non, la stérilité n'est pas héréditaire"

Alphonse Allais (Ibidem)

"Voici le printemps, et mon arbre généalogique n'est pas encore en fleurs"

Erik Satie

La minéralisation ou la calcification des parties molles du corps humain peut être considérée comme une partie d'un processus dégénératif permanent qu'on appelle généralement "le vieillissement".

Être un exemple de l'ingéniosité technologique et de bio-engeneering excellent sans égal, même le dépérissement de ce "*corpus humanum* " ne peut guère être considéré comme un événement arbitraire ou passif. Au contraire, la précipitation du calcium est réglée très strictement par un jeu d'ensembles intriguant des facteurs induisants et des protéines inhibiteurs. Ainsi, il a été réglé que nous ne changeons pas lors de notre jeunesse en un pilier de craie, mais que par contre, nous subissons un processus lent et progressif de minéralisation. Mais quand ce opus régularisant brilliant ne fonctionne plus de façon optimale, nous sommes confrontés à un groupe de maladies particulières: les affections de minéralisation ectopique.

Un des archétypes de ces affections est le pseudoxanthome élastique ou le PXE, dans lequel la calcification ectopique des fibres élastiques cause des symptômes des yeux, de la peau et cardio-vasculaires. Quoique le PXE ait été décrit pour la première fois il y a deux siècles, la maladie - comme c'est le cas pour beaucoup d'affections génétiques - reste entourée d'un voile mystérieux. Le but de cette thèse est donc d'apporter une contribution aux caractéristiques histopathologiques, cliniques et moléculaires de cette affection fascinante.

Par la caractérisation clinique soigneuse d'un groupe de patients ayant le PXE, suivis dans le Centre de Génétique Médicale Gantois, nous avons eu la possibilité d'accentuer l'intérêt de certaines caractéristiques cliniques, comme les infarctus cérébraux et l'athérosclérose. En plus, nous avons identifié des caractéristiques phénotypiques nouvelles chez les patients et chez les porteurs/porteuses hétérozygotes, parmis lesquels les calcifications des organes du ventre et le microlithiase testiculaire. De plus, l'existence d'un phénotype partiel ou indulgent chez les porteurs/porteuses du PXE a été abordé et nous avons démontré que ces individus courrent un risque cardio-vasculaire plus important, conjointement aux caractéristiques ophtalmologiques restreintes s.f.d. comets et comet tails.

Dans une étude pilote d'un groupe de plus de 200 patients ayant eu un infarctus cérébral, nous avons retrouvé – en utilisant une analyse moléculaire des hotspots du gène *ABCC6* – un nombre plus élevé de mutations hétérozygotes dans ce gène par rapport à un groupe de contrôle. Cet argument complémentaire confirme que les hétérozygotes ont un plus grand risque concernant les maladies cardio-vasculaires; de plus, cette étude introduit le gène *ABCC6* dans la recherche étiologique des infarctus cérébraux.

Par des études locales et multi-centres, cette thèse a aussi contribué à la description et à l'élargissement du spectre des mutations du gène *ABCC6*, de même que l'exclusion pertinente des corrélations entre le phénotype et le génotype. La stratégie d'analyse moléculaire, laquelle a

été utilisée jusqu'ici pour l'analyse du gène *ABCC6*, démontre son efficacité, avec un rapport excellent entre les coûts et les profits et un ratio de détection des mutations qui est le plus élevé ayant été rapporté dans la littérature récente. En plus, la discussion persistante concernant le mode d'hérédité du PXE et en particulier si une forme dominante existe, a été examiné.

Pendant le suivi des patients ayant le PXE, nous avons utilisé des techniques nouvelles pour examiner le fond de l'œil, comme l'auto-fluorescence et la lumière infrarouge qui nous permettent de créer une image plus claire, d'où une amélioration des possibilités diagnostiques lors des lésions ophtalmologiques subtiles. En collaboration avec Moorsfield Eye Hospital de Londres, nous avons abordé également l'intérêt sur des anormalités electrophysiologiques de la rétine, dans lequel ont pu discerner trois phénotypes différents.

Au cours des recherches, nous avons eu la possibilité d'identifier une nouvelle affection comme une entité séparée et de la caractériser, aussi bien cliniquement qu'au niveau moléculaire. Cette nouvelle maladie récessive a été appelé le PXE-like syndrome, à cause des nombreuses similarités avec le PXE classique. Nous avons trouvé que la maladie est causée par des mutations dans le gène *GGCX,* qui code pour un gamma-carboxylase. Cet enzyme joue un rôle important dans le cycle métabolique de la vitamine K (VK). De cette façon, nous avons pu introduire la VK, mais aussi les protéines qui sont dépendantes de cette vitamine pour leur fonctionnement - comme différents inhibiteurs de calcification - dans la pathogénèse du PXE-like syndrome, mais aussi dans le PXE.

En utilisant plusieures expériences immunohistochemiques et ELISA, nous avons pu démontrer que différents inhibiteurs de calcification étaient inactif ou non-actif dans ces deux syndromes, ce qui occasionne la calcification ectopique. Ces constatations sont valables jusqu'aux mutations dans le gène *GGCX* dans le PXE-like syndrome. Dans le PXE, l'explication pour ces protéines déficientes a été démontrée par des quantités très faibles de la VK - un facteur essentiel pour la carboxylation des protéines dans le cycle métabolique de la VK - dans le sang des patients avec le PXE. Bien que la relation entre le transporter muté ABCC6 et ces quantités faibles de la VK reste à présent imprécis, la séduisance reste de faire l'hypothèse que soit la VK, soit un de ses précurseurs ou métabolites associés, est transporté par le transporter ABCC6. En outre, ces constatations ouvrent de nouvelles perspectives pour la suppléance de la VK comme traitement pour le PXE.

Comme décrit ci-dessus, les résultats de cette recherche ont permis un élargissement de la connaissance clinique et moléculaire du PXE et de ses affections apparentées. De nouvelles routes ont été ouvertes pour la recherche fondamentale et appliquée dans la branche de la minéralisation ectopique. Par dessus tout, ce sont les patients et leur famille qui profitent de nos résultats, grace à un diagnostic moléculaire plus efficace, un suivi ponctuel et la perspective d'un traitement pour leur affection contraignante.

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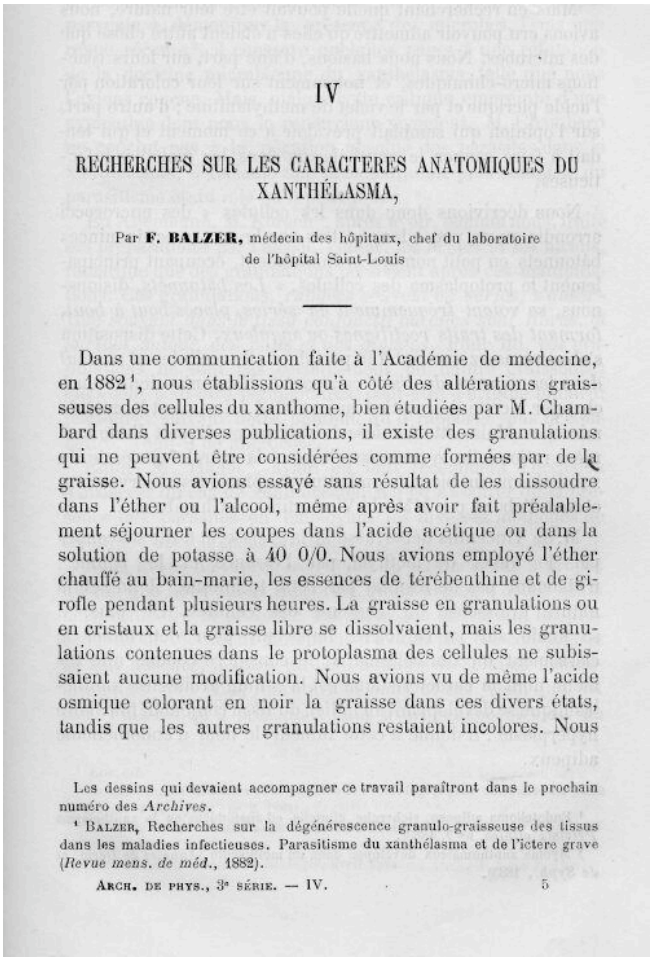
Appendix

BALZER, Félix. **Recherches sur les caractères anatomiques du xanthélasma**. Archives de physiologie normale et pathologique. 1884;4: 5-80



IV

RECHERCHES SUR LES CARACTERES ANATOMIQUES DU XANTHÉLASMA,

Par **F. BALZER,** médecin des hôpitaux, chef du laboratoire de l'hôpital Saint-Louis

Dans une communication faite à l'Académie de médecine, en 1882 [1], nous établissions qu'à côté des altérations graisseuses des cellules du xanthome, bien étudiées par M. Chambard dans diverses publications, il existe des granulations qui ne peuvent être considérées comme formées par de la graisse. Nous avions essayé sans résultat de les dissoudre dans l'éther ou l'alcool, même après avoir fait préalablement séjourner les coupes dans l'acide acétique ou dans la solution de potasse à 40 0/0. Nous avions employé l'éther chauffé au bain-marie, les essences de térébenthine et de girofle pendant plusieurs heures. La graisse en granulations ou en cristaux et la graisse libre se dissolvaient, mais les granulations contenues dans le protoplasma des cellules ne subissaient aucune modification. Nous avions vu de même l'acide osmique colorant en noir la graisse dans ces divers états, tandis que les autres granulations restaient incolores. Nous

Les dessins qui devaient accompagner ce travail paraîtront dans le prochain numéro des *Archives*.

[1] BALZER, Recherches sur la dégénérescence granulo-graisseuse des tissus dans les maladies infectieuses. Parasitisme du xanthélasma et de l'ictère grave (*Revue mens. de méd.*, 1882).

ARCH. DE PHYS., 3e SÉRIE. — IV. 5

Curriculum Vitae

"It does tell us something. Though I have no idea what."

Gregory House, MD

1. Personalia

NAME	**VANAKKER**
FIRST NAME	Olivier M.F.M.A
DATE & PLACE OF BIRTH	10 february 1978, Ghent
NATIONALITY	Belgian
MARITAL STATUS	Married to Liesbeth De Groote Father of Simon
HOME ADRESS	David Tenierslaan 1 9051 Sint-Denijs-Westrem Tel: +32-9-3885223
WORK ADRESS	Department of Paediatrics & Center for Medical Genetics Ghent University Hospital De Pintelaan 185 9000 Ghent Tel +32-9-3326598 Fax +32-9-3324970 E-mail: olivier.vanakker@ugent.be Web: www.cmgg.be
LANGUAGES	Dutch mother tongue English fluent French fluent German basics

2. Training

June 2001: Certificate "electrocardiography", Ghent University, Belgium

Graduated as Medical Doctor (great distinction) in 2003, Ghent University, Belgium

October 2003 – present : trainee in paediatrics

October 2003 – september 2007: aspirant research grant of the Fund for Scientific Research Flanders (FWO): "Unravelling the molecular-genetic basis of hereditary disorders of elastin" (Promotor ; Anne De Paepe, MD, PhD ; co-promotor : Dirk Matthys, MD, PhD)

June 2007 – present : assistant coordinator of the EuroPXE research consortium

October 2007 – present : scientific collaborator at the CMG Ghent

October 2007 : Certificate « European Paediatric Life Support », Ghent University, Belgium

Reviewer for *The American Journal of Medical Genetics*, *European Journal of Medical Genetics*, *Diagnostic Molecular Pathology*, *European Journal of Human Genetics, European Journal of Paediatrics, The Journal of Pathology, Cerebrovascular Diseases, Clinica Chimica Acta*, *Journal of Paediatrics*, *Molecular Biology and Evolution, Proteomics* and *Journal of Investigative Dermatology*.

Reviewer for the 3rd International Symposium on Bio- and Medical Informatics and Cybernetics, Orlando, Florida, USA, July 10-13th 2009.

Subinvestigator for the EPI-ROTA-111426 study (Hospital-based, prospective case-control study to assess the efficacy of the weakened human rotavirus vaccination (Rotarix™) of GSK Biologicals against community-acquired severe rotavirus gastro-enteritis in hospitalised children born after 01.10.1006 in Belgium).

3. Publications

A/ Papers

1/ **Vanakker O**, De Baets F, Franckx H, Matthys D. Muco-viscidose en fysieke training: nut van een kortdurend trainingsprogramma. Tijdschr Geneesk 2001; 57(3): 190-194

2/ Verhaaren HA, **Vanakker O**, Van Coster R, De Wolf D, François K, Matthys D. Use of an event recorder in the decision for pacemaker implantation in a child with syncope. Eur J Ped 2002; 161: 267-269

3/ Verhaaren HA, **Vanakker O**, De Wolf D, Suys B, François K, Matthys D. Left ventricular outflow obstruction in rhabdomyoma of infancy: meta-analysis of the literature. J. Ped 2003; 143(2):258-263

4/ **O. M. Vanakker**, D. Voet, M. Petrovic, F. Van Robaeys, B.P. Leroy, P. Coucke, A. De Paepe. Visceral and testicular calcifications as part of the phenotype in Pseudoxanthoma Elasticum: ultrasonographical findings in Belgian patiënts and healthy carriers. Br J Radiol 2006 Mar;79(939):221-5

5/ Isabelle Audo*, **Olivier M Vanakker***, Bart P Leroy,Anthony G Robson, Alaric Smith, Sharon A Jenkins, Paul J Coucke, Alan C Bird, Anne De Paepe, Graham E Holder, Andrew R Webster. Pseudoxanthoma elasticum with generalized retinal dysfunction, a common finding? IOVS 2007 Sep;48(9):4250-6
(* joint first authors)

6/ **Olivier M. Vanakker**, Ludovic Martin, Dealba Gheduzzi, Bart P. Leroy, Bart Loeys, Veronica I.Guerci, Dirk Matthys, Sharon F. Terry, Paul J. Coucke, Ivonne Pasquali-Ronchetti, Anne De Paepe. Pseudoxanthoma Elasticum-like phenotype with cutis laxa and multiple coagulation factor deficiency represents a separate genetic entity. J Invest Dermatol 2007;127:581-87

7/ **O.M. Vanakker**, B.P. Leroy, P.Coucke, P. Van Acker, D. Matthys, S.F. terry, E. Pfendner, B. Loeys, A. De Paepe. Novel clinico-molecular insights in Pseudoxanthoma Elasticum provide an efficient molecular screening method and a comprehensive diagnostic flowchart. Hum Mutat 2008;29:205

8/ Ellen G. Pfendner*, **Olivier M. Vanakker*,** Sharon F. Terry, Sophia Vourthis, Patty McAndrew, Monica R. McClain, Sarah Fratta, Anna-Susan Marais, Susan Hariri, Paul J. Coucke, , Michele Ramsay, Denis Viljoen, Anne De Paepe, Jouni Uitto, Patrick F. Terry, Lionel G. Bercovitch. Mutation Detection in the *ABCC6* Gene and Analysis in a Large International Case Series Affected by Pseudoxanthoma Elasticum. J Med Genet 2007;44:621-628
(* joint first author)

9/ **Vanakker O**, De Paepe A. Pseudoxanthoma elasticum: een metabole bindweefselziekte. Tijdschr Geneesk 2008;3:135-140

10/ N. Turfaner, **O.M. Vanakker**, R. Bayar, C. Islak, F. Sipahioglu. A variant of Ehlers-Danlos manifesting as manic episode. Cerrahpasa Med Rev 2007;5(1):45-49

11/ **Vanakker O**, Malfait F, De Paepe A. Osteogenesis Imperfecta. Nederlands Tijdschrift voor Calcium en Bothomeostasis 2008;3:69-73

12/ Costrop L, **Vanakker OM**, Van Laer L, Le Saux O, Martin L, Chassaing N, Pasquali-Ronchetti I, Coucke PJ, De Paepe A. Multiplex ligation-dependent probe amplification improves mutation detection rate in pseudoxanthoma elasticum. Submitted to Hum Mutat.

B/ Abstracts

Poster presentations

1/ Vanakker O, De Baets F, Franckx H, Matthys D. Cystic fibrosis and physical training: Effects of a short-term training program. North American Society for Pediatric Exercise Medicine Conference; Aspen (USA), August 10-12th 2000

2/ **Vanakker O**, Verhaaren HA. Rhabdomyomas in infancy. Prenatal diagnosis of a severe LVOT obstruction treated by partial resection and review of literature. Annual meeting of the Belgian Paediatric Society; Essene, March 15th 2002

3/ **O.M. Vanakker**, P. Coucke, B.P. Leroy, D. Voet, E. De Baere, P. Van Acker, A. De Paepe. Pseudoxanthoma elasticum: mutation analysis and phenotypical characterization in Belgian patients and heterozygous carriers. Annual meeting of the American Society of Human Genetics, Toronto, Canada, October 26th – 30th 2004

4/ **O.M. Vanakker**, P. Coucke, B.P. Leroy, D. Voet, E. De Baere, P. Van Acker, A. De Paepe. Pseudoxanthoma elasticum: mutation analysis and phenotypical characterization in Belgian patients and heterozygous carriers. Annual meeting of the Department of Internal Medicine, Ghent University Hospital, Ghent, January 20th 2005

5/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, I. Pasquali-Ronchetti, A. De Paepe. Pseudoxanthoma elasticum-like disorder with generalized cutis laxa and clotting deficiency represents a novel genetic entity. Annual meeting of the European Society of Human Genetics, Amsterdam, the Netherlands, May 6-9th 2006

6/ B. Callewaert, B. Albrecht, B. Loeys, G. Gillessen-Kaesbach, I. Hausser, **O. Vanakker**, P.J. Coucke, Z. Urban, A. De Paepe. Two novel mutations in the ELN gene in patients with autosomal dominant cutis laxa and systemic manifestations. Annual meeting of the European Society of Human Genetics, Amsterdam, the Netherlands, May 6-9th 2006

7/ B. Callewaert, B. Albrecht, B. Loeys, G. Gillessen-Kaesbach, I. Hausser, **O. Vanakker**, P.J. Coucke, Z. Urban, A. De Paepe. Two novel mutations in the ELN gene in patients with autosomal dominant cutis laxa and systemic manifestations. 34th annual meeting of the Belgian Society of Paediatrics, Brugge, March 17-18th 2006

8/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, I. Pasquali-Ronchetti, A. De Paepe. Pseudoxanthoma elasticum-like disorder with generalized cutis laxa and clotting deficiency represents a novel genetic entity. Annual meeting of the Department of Internal Medicine, Ghent University Hospital, Ghent, March 30th 2006

9/ I.S. Audo, **O.M. Vanakker**, B.P. Leroy, A.G. Robson, P.J. Coucke, A.C. Bird, A. De Paepe, G.E. Holder, A.R. Webster. Pseudoxanthoma elasticum with generalized retinal dysfunction: a common finding? Annual meeting of the Association for Reserach in Vision and Ophthalmology (ARVO), May 4th 2006

10/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, I. Pasquali-Ronchetti, A. De Paepe. Pseudoxanthoma elasticum-like disorder with generalized cutis laxa and clotting deficiency represents a novel genetic entity. Elastin Meeting 2006, Grenoble, France, July 9-12th 2006

11/ Gheduzzi D, Annovi G, **Vanakker OM**, Martin L, Leroy BP, Loeys B, Matthys D, Coucke PJ, Schurgers L, de Paepe A, Pasquali-Ronchetti I. Elastic fiber calcification and pseudoxanthoma elasticulm-like phenotype in patients with vitamin K-dependent deficiency of coagulation factors. Elastin Meeting 2006, Grenoble, France, July 9-12th 2006

12/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. Mutations in the gamma-carboxylase gene GGCX cause a novel pseudoxanthoma elasticum-like disorder. Annual meeting of the Department of Internal Medicine, Ghent University Hospital, Ghent, March 14th 2007

13/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. Mutations in the gamma-carboxylase gene GGCX cause a novel pseudoxanthoma elasticum-like disorder. Annual meeting of the European Society of Human Genetics, Vienna, Austria, June 16-19th 2007

14/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. A common pathogenetic role for vitamin-K dependent inhibitors of calcification in PXE and the PXE-like syndrome: novel insights in ectopic mineralization. Annual meeting of the American Society of Human Genetics, San Diego, US, October 23th – 27th 2007

15/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. A common pathogenetic role for vitamin K-dependent inhibitors of calcification in PXE and the PXE-like syndrome: novel insights in ectopic mineralization. Annual meeting of the Department of Internal Medicine, Ghent University Hospital, Ghent, March 18th 2008

16/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. A common pathogenetic role for vitamin K-dependent inhibitors of calcification in PXE and the PXE-like syndrome: novel insights in ectopic mineralization. Annual meeting of the Belgian Society of Human Genetics, Louvain, Belgium, April 25th 2008

17/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. A common pathogenetic role for vitamin K-dependent inhibitors of calcification in PXE and the PXE-like syndrome: novel insights in ectopic mineralization. Annual meeting of the European Society of Human Genetics, Barcelona, Spain, May 31st- June 3th 2008

18/ Y. Le Corre, L. Martin, **O. M. Vanakker**, L. Schurgers, D. Gheduzzi, C. Vermeer, I. Pasquali-Ronchetti, P. Coucke, A. De Paepe. Anomalies leading to the mineralization of elastic fibers in pseudoxanthoma elasticum and the PXE-like syndrome. Submitted to congrés annuel de recherché dermatologique, Toulouse, France, September 11th-13th

19/ P. D. Turnpenny, **O. M. Vanakker**, L. Costrop, A. de Paepe, L. Schurgers, R. Florijn, F. M. Pope, M. James, S. Tomkins, P. Newman, S. Ellard, E. Young, M. L. P. Robert. A novel, autosomal dominant, Pseudoxanthoma Elasticum-like phenotype in a five-generation family. Annual meeting of the American Society of Human Genetics, Philadelphia, US, November 11-15th 2008

20/ Quaglino D, Gheduzzi D, Tarugi P, Guerra D, Roggiani J, Boraldi F, Annovi G, **Vanakker O**, Coucke P, De Paepe A, Ronchetti I. Correlation between ApoE polymorphism and severity of cardiovascular manifestations in pseudoxanthoma elasticum (PXE). Annual meeting of the Italian Society for the Study of Connective Tissues, Pavia, Italy, November 6-7th 2008

21/ L. Costrop, **O. M. Vanakker**, P. Coucke, L. Martin, N. Chassaing, I. Pasquali-Ronchetti, A. De Paepe. Multiplex Ligation-Dependent Probe Amplification refines Molecular Diagnosis in Pseudoxanthoma Elasticum. Annual meeting of the Belgian Society of Human Genetics, Brussels, Belgium, February 13th 2009

22/ L. Costrop, **O. M. Vanakker**, P. Coucke, L. Martin, N. Chassaing, I. Pasquali-Ronchetti, A. De Paepe. Multiplex Ligation-Dependent Probe Amplification refines Molecular Diagnosis in

Pseudoxanthoma Elasticum. Annual meeting of the European Society of Human Genetics, Vienna, Austria, May 23-26th 2009

23/ **O. M. Vanakker**, B. P. Leroy, L. J. Schurgers, I. Pasquali-Ronchetti, P. J. Coucke, A. De Paepe. An atypical case of pseudoxanthoma elasticum with abdominal cutis laxa: evidence for a clinical disease spectrum. Submitted to the Annual meeting of the American Society of Human Genetics, Honolulu, Hawaii, October 20-24th 2009

Oral presentations

1/ **Vanakker O**. Pseudoxanthoma Elasticum: a clinical and molecular review. Scientific staffmeeting Paediatrics, Ghent University Hospital, November 18th 2003

2/ **Vanakker O.** Congenital Adrenal Hyperplasia: clinical and genetic aspects. Stafmeeting CMG, Ghent University Hospital, January 20th 2004

3/ **O.M. Vanakker**, B.P. Leroy, P. Coucke, D. Voet, M. Petrovic , A. De Paepe Visceral calcifications as part of the phenotype in Pseudoxanthoma Elasticum: ultrasonographical findings in Belgian patients and heterozygous carriers. PXE Reserach Meeting, Bethesda, US, October 14-15th 2004.

4/ **O.M. Vanakker**. Pseudoxanthoma elasticum: a hereditary metabolic multi-systemic disease. Scientific staff meeting of the Department of Ophthalmology, Ghent University Hospital, October 29th 2004

5/ **O.M. Vanakker.** Should dysmorphologists know about pseudoxanthoma elasticum? Regional dysmorphology meeting AZ-VUB, Brussels, Belgium, November 5th 2004

6/ **O.M. Vanakker.** The Ghent PXE clinic. PXE Research Meeting, Ghent, Belgium, November 26-27th 2004

7/ **O. Vanakker.** Pseudoxanthoma elasticum: neurovascular involvement in a hereditary multi-systemic disorder. Scienttific Staff Meeting of the Department of Neurology, Ghent University Hospital, Ghent, March 15th 2005

8/ **O. Vanakker**, B. Loeys, B. Leroy, P. Coucke, A. De Paepe. What should paediatricians know about Pseudoxanthoma Elasticum? 34th annual meeting of the Belgian Society of Paediatrics, Brugge, march 17-18th 2006

9/ **O. Vanakker.** Unravelling the pathogenesis of elastic fiber disorders: pseudoxanthoma elasticum – research in progress. Scientific staff meeting of the Center for Medical Genetics, Ghent University Hospital, May 23th 2006

10/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, I. Pasquali-Ronchetti, A. De Paepe. Pseudoxanthoma elasticum-like disorder with generalized cutis laxa and

clotting deficiency represents a novel genetic entity. Annual meeting of the American Society of Human Genetics, New Orleans, US, October 9th – 13th 2006

11/ **O. Vanakker.** Pseudoxanthoma elasticum and related disorders. Geneskin workshop, Ghent, Belgium, December 6th 2006

12/ **O. Vanakker**. Novel clinico-molecular and pathophysiological insights in pseudoxanthoma elasticum and related disorders: reserach in progress. Scientific meeting of the Department of Paediatrics, Ghent University Hospital, January 16th 2007

13/ **O.M. Vanakker**. A novel PXE-like disorder, caused by mutations in the GGCX gene, suggests a pathogenetic role for vitamin K-dependent inhibitors of calcification in PXE. Gordon conference on Elastin and Elastic fibers, Biddeford, US, July 29th – 3d 2007

14/ **O.M. Vanakker**, , L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. The role of vitamin K-dependent proteins in the pathogenesis of PXE and PXE-like disorders. Mini-symposium "recent progress in cardiovascular research", Ghent, belgium, March 13th 2007

15/ **O.M. Vanakker.** Hereditary connective tissue disorders associated with stroke. Scientific staff Meeting of the Department of Neurology, Ghent University Hospital, March 29th 2007

16/ **O. Vanakker**. Novel clinico-molecular and pathophysiological insights in pseudoxanthoma elasticum and related disorders: reserach in progress. Scientific meeting of the Center for Medical Genetics, Ghent University Hospital, April 3d 2007

17/ **O.M. Vanakker**, L. Martin, D. Gheduzzi, B.P. Leroy, B. Loeys, P.J. Coucke, S.F. Terry,L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. Mutations in the gamma-carboxylase gene GGCX cause a novel pseudoxanthoma elasticum-like disorder. Annual meeting of the Belgian Society of Human Genetics, Marcinelle, Belgium, April 20th 2007

18/ **O.M. Vanakker.** Pseudoxanthoma elasticum as an example of patient care and research in genetics. Teaching class Medicine Students, Ghent University, June 2007

19/ **O.M. Vanakker**. Novel insights in the pathogenesis of pseudoxanthoma elasticum and related disorders. PXE Research Meeting, Budapest, Hungary, September 13-14th 2007

20/ **O.M. Vanakker**, , L. Martin, P.J. Coucke, L.J. Schurgers, C. Vermeer, I. Pasquali-Ronchetti, A. De Paepe. Deficient inhibitors of calcification act as common final pathway in PXE and the PXE-like syndrome. 1st world congress on genodermatology, Maastricht, the Netherlands, November 8th 2007

21/ L. Martin, **O. Vanakker**, L. Schurgers, B. Leroy, B. Loeys, P. Coucke, C. Vermeer, A. De Paepe. La calcification des fibres élastiques au cours des pseudoxanthomes élastiques classique et variant, respectivement liés aux gènes ABCC6 et GGCX, est associée à un déficit en inhibiteurs de la minéralisation. Les journées dermatologiques de Paris, France, December 4-8th 2007

22/ P. D. Turnpenny, **O. M. Vanakker**, L. Costrop, A. de Paepe, L. Schurgers, R. Florijn, F. M. Pope, M. James, S. Tomkins, P. Newman, S. Ellard, E. Young, M. L. P. Robert. A novel, autosomal dominant, Pseudoxanthoma Elasticum-like phenotype in a five-generation family. Annual meeting of the European Society of Human Genetics, Barcelona, Spain, May 31st- June 3th 2008

23/ **O.M. Vanakker.** Vitamin K. Novel insights into an ancient molecule. Neonatology staff meeting., Ghent University Hospital ,February 19th 2009

24/ **O.M. Vanakker.** Vitamin K as a therapeutic option in PXE. Fact or fiction? PXE research meeting, University Hospital of Angers, France, May 12th 2009

4. Various

- Counsellor for medical school student theses:

"The role of ABC trasporters in human physiology and pathophysiology" (2006-2007)
"Genetic determinants of cerebrovascular accidents" (2007-2008)
"Vitamin K revisited: novel insights in an ancient molecule" (2007-2008)
"Novel insights into the pathophysiology, clinical characteristics and treatment of fibromuscular dysplasia" (2007-2008)
"Genetic determinants of intracranial aneurysms" (2008-2009)
"The use of vitamin K in treatment of osteoporosis: from vitamin supplement to therapeutic drug" (2008-2009)

Dankwoord

Waar is de weg ? Er is geen weg.
Op naar het onbekende!

Goethe (*Faust)*

Beste lezer, sta mij toe mijn dankwoord te starten met een uitspraak van één van mijn grote voorbeelden – voor sommigen zal alles nu misschien duidelijk worden –, met name de Catalaanse surrealist Salvador Dalì. In 1963 sprak hij in een interview de onsterfelijke woorden: "*There are some days when I think I'm going to die from an overdose of satisfaction.*" Welnu, een soortgelijk gevoel overvalt mij op het ogenblik van het schrijven van dit dankwoord. Het ei is gelegd, de laatste borstelstreken hebben het canvas beroerd. Perfectie bestaat niet, zoals mijn mentor mij altijd zei, maar je moet het altijd trachten na te streven. Bij deze, denk ik dan bij mezelf. Het resultaat van vier jaar bloed, zweet en tranen ligt nu in uw handen, klaar om doorbladerd te worden. En wees niet bang, laat u niet afschrikken om behalve de laatste bladzijden – het dankwoord, de samenvatting en het curriculum – ook de eerste vier hoofdstukken van dit werk door te nemen. Ze zijn voor u geschreven, met evenveel liefde en strevend naar verstaanbaarheid, als de laatste deeltjes van dit boek. Dat u de referentielijst niet van begin tot einde napluist, wil ik u gerust vergeven.

Zoals u wellicht al gemerkt heeft of zal merken bij uw avond- en nachtelijke literatuur van dit werk, is het behalve de weergave van mijn academische activiteiten tot op heden ook gelardeerd met een aantal citaten, noem het intellectuele doordenkertjes, waar u ongetwijfeld nog uren genoegen zult aan beleven. Alle methodes zijn goed om meer mensen dan alleen de jury aan te zetten om het boek van voor naar achter uit te pluizen.

Research, beste lezer, is vaak een moeilijke, maar altijd uitdagende opdracht, soms op de tast en vaak met vallen en opstaan. Goethe had misschien wel dit soort van professionele tijdsbesteding in zijn achterhoofd, want wetenschappelijk onderzoek *pur sang* is het verleggen van grenzen, het naast-de-betreden-paadjes-lopen, het zelf aanleggen van klinkerweggetjes doorheen het tot dan toe onbekende. Het is een vak dat velen tot de verbeelding spreekt, zeker als je interesses zich duidelijk manifesteren in de zogenaamd grote maatschappelijke problemen van deze tijd: AIDS, kanker, etc. Wanneer je het in je hoofd haalt om een zeldzame ziekte, één van de "weeskindjes van de pathologie", te bestuderen, pleegt de reactie hierop voornamelijk van de aard: "En hoe is het nog met de kinderen?" te zijn. Hoewel ik sinds kort al een wat zinniger antwoord kan geven op dergelijke vragen, blijft het moeilijk om anderen te overtuigen van het nut en de "*added value*" die de studie van zeldzame ziekten met zich meebrengt. Vandaar dat de niet-geringe gravitas van de taak - het ophouden van een al dan niet vakkundig geveinsde interesse - waarvan zij die mij doorheen dit werk hebben gesteund zich hebben gekweten, meer dan ooit een welgemeend dankjewel verdiend.

Beste lezer, zo'n dankwoord brengt met zich mee dat enkele mensen - de happy few - speciale aandacht krijgen. Gedurende de voorbij jaren heb ik de eer gehad met heel veel leuke, sympathieke en – laat ons eerlijk zijn – soms ook minder sympathieke mensen samen te werken, te discussiëren, te lachen, etc. Vermoedelijk teveel om hier allemaal op te noemen en laat het die ene persoon die dit lijstje overloopt en zich vergeten voelt duidelijk zijn: het was niet mijn

bedoeling. Ongetwijfeld ben je tijdens het schrijven van dit werk vaak in mijn gedachten geweest en ik maak het goed tijdens de receptie door je een extra *hors d'oeuvreke* te schenken…

Laat mij toe te starten met diegenen die voor de inhoud van dit werk onontbeerlijk zijn geweest. Die mensen die ik steeds bereid vond voor een nieuwe studie, een nieuw onderzoek, alwéér een bloedafname. "Mijn" PXE patiënten en hun familie. Weet dat zonder jullie input dit werk niet tot stand zou zijn gekomen. Ik dank jullie van ganser harte voor jullie bereidwilligheid en interesse. Pour mes patients francophones: un grand merci de tout mon coeur. For my overseas patients: my warmest feelings of gratitude for the many collaborations so far.

Beginnen met doctoreren is zoals duiken van de hoogste springplank: je weet niet waar je aan begint als je er voor staat maar pas wanneer je op de rand balanceert, licht naar het water hellend, dan besef je: ik wil terug naar beneden. En zoals mijn befaamde driedubbele salto met halve draai en dubbele twist ook niet van in het begin volledig goed zat, zo zijn er ook voor een doctoraat mensen nodig om je salto's, draaien en twists – in een wereld die niet de jouwe is - te begeleiden en in goede banen te leiden. Ik ben in de eerste plaats enorm veel dank en erkenning verschuldigd aan mijn promotor, prof. dr. Anne De Paepe. Dank om in mij iemand te zien die deze opdracht tot een goed einde zou kunnen brengen en om mij toe te laten tot de getalenteerde en gedreven groep die het Centrum Medische Genetica in het algemeen en het bindweefselteam in het bijzonder is. Ik denk dat ik u zonder enige schroom mijn mentor kan noemen. Met vaste – en soms harde – hand hebt u mij weten te leiden rondom de hindernissen en valkuilen die inherent zijn aan elke vorm van wetenschappelijk onderzoek zodat de uitkomst niet werd gecompromitteerd. Geleidelijk aan – en vaak zonder ik er erg in had – hebt u mij laten kennis maken met alle facetten van research – onderzoek is meer dan enkel proefjes uitvoeren – en mij hierin meer verantwoordelijkheden gegeven. Als het goed is, is het goed en als het slecht is, is het slecht; een houding die ik tot op de dag van vandaag, in een wereld waar ik tot de ontdekking ben gekomen dat een eenduidige mentaliteit geen evidentie is, nog steeds enorm apprecieer.

Daarnaast wens ik ook mijn co-promotor, prof. dr. Dirk Matthys, te danken om mij – als jonge assistent kindergeneeskunde – in de richting van genetica te leiden. In een zeer klinisch georiënteerde discipline als pediatrie is een combinatie met topresearch geen evidentie – een mentaliteit waar ik op dit ogenblik, tijdens het vervolledigen van mijn klinische opleiding, dagdagelijks mee wordt geconfronteerd – doch zijn enthousiasme heeft mij doen opteren mijn academische carrière te starten met dit doctoraat.

Verder wil ik de overige hardcore leden van het bindweefselteam uitbundig danken. Prof. dr. Paul Coucke, beste Paul, voor het openzetten van de deuren van het bindweefsellabo, de begeleiding, de zomerse paëlla en de zwoele salsa (dit laatste niet als danspartner, welteverstaan). Dr. Bart Leroy, beste Bart, voor de bemoedigende woorden en opbouwende kritiek tijdens het schrijven van mijn eerste papers, de vakkundige introductie in de geheime kamers van de oftalmologie, voor het keuvelen, de ritjes in de Lotus (ik heb er nog hartkloppingen van) en bovenal de vriendschap. Jawel beste lezer, samen met Bart en Paul tijdens de paastijd om 1 uur 's nachts werken aan een paper, enkel gaande gehouden door het verorberen van chocoladen paaseieren: op zo'n momenten besef je wat vriendschap is...
Dr. Bart Loeys, beste Bart 2 ;-), je kennis en expertise in de klinische en moleculaire genetica hebben me ongelooflijk veel geleerd. Dank voor het aangename gezelschap op congres,

alhoewel ik moet zeggen – ik heb je dit nog niet bekend – ik haat doughnuts en ik verafschuw om 's morgens op zoek te moeten gaan naar een ontbijt i.p.v. in het hotel te eten. Volgende keer kies ik dus het hotel! En dan de meiskes. Where does one begin... Sophie, Soppie voor de vrienden, je bent de animatie in het bindweefsellabo, de bindweefselstaf, de bindweefselcongressen en zoveel meer. Dankjewel voor de sfeer, de aangename babbel, de smoelentrekkerij op momenten dat het absoluut niet gepast was. Sofie, wie anders kon het zich veroorloven met een halve meter biochemie-dossiers ter nazicht af te komen en mij toch nog een glimlach te ontlokken.
Marjolein, je bent één van de laatsten die in onze besloten kring is toegelaten. Het verwondert mij keer op keer hoe snel je je aan de anderen hebt aangepast. Hoe snel stijl kan plaats maken voor..., nu ja. In elk geval, verander niets, je past perfect binnen het profiel van een bindweefseler.
Ik hoop, beste meisjes, dat we samen nog vele after-wetenschapsdag dinners mogen hebben, and we can feast like there is no tomorrow...

Ongeveer een jaar geleden werd ik, na een lange zoektocht, bijgestaan door een jongedame die nu actief binnen de dagelijkse werking van de PXE research groep functioneert. Beste Laura, ik heb je eerder al verteld dat mijn eerste indruk van jou er één was van "we gaan dit jonge veulen serieus in toom moeten houden". De resultaten die je tot nog toe hebt behaald tonen – behalve de kwaliteit van de door mij ontworpen studies ;-) – aan dat we ons niet hebben vergist in jou. Je bent een aanwinst voor deze groep en ik dank je oprecht voor het vele werk dat je reeds hebt verzet en waardoor je meehelpt het onderzoek binnen ons domein in stand te houden.

Tijdens mijn doctoraat ben ik ongeveer 7 maal van bureau veranderd, een record in onze dienst, maar het laatste anderhalf jaar had ik het geluk mijn dagdagelijkse beslommeringen te mogen delen met dr. Bert Callewaert en dr. Kristien Hoornaert. Beste Bert en Kristien, dank voor het gezelschap, de Delhaize-sfeer door de massa voedingswaren van Kristien en de sloten koffie van Bert. En Bert, coke rules!

Mijn eerste schuchtere pasjes in het bindweefsellabo zouden een aaneenschakeling geweest zijn van (nog meer) uitschuivers en valpartijen, ware het niet voor de hulp, het begrip en het onvermoeibare enthousiasme van de talrijke laboranten. In een tijd waarin vechten voor een centrifuge in het toenmalige K5 labo nog werd aanzien als volwaardige topsport en sequeneren – wegens het toen nog hoge science fiction gehalte van een sequencing robot een ware marathon van pipetteerbewegingen – een grondige opwarming van de duimspieren vereiste, loodsten zij mij met engelengeduld doorheen het moeizame proces van mijn eerste PCR's, dHPLC resultaten en sequenties. Ik herinner mij nog de warme gevoelens die mij overvielen bij het zien van mijn eerste PCR resultaat: een maagdelijk zwarte gelfoto, geen wit vlekje geamplificeerd DNA op te zien. Maar zie, jullie kundigheid is dan toch een beetje op mij afgestraald. Petra - de mater familias van het bindweefsellabo, die altijd alle producten weet staan in elk hoekje van welke diepvriezer ook te velde -, Renee, Karen, Inge, Chantal, Nilgul en alle anderen: ik ben jullie dankbaar voor het geduld, de ondersteuning, het gekwebbel (ik zou het woord 'geroddel' niet over mijn lippen kunnen krijgen ;-) en de vriendschap.

Mijn klinische activiteiten in het CMGG brachten mij onherroepelijk ook in contact met de medici en paramedici die de genetische kliniek, vaak fluitend en niet gespeten van enige zwier, draaiende hielden en patiënten (en soms ook artsen en doctoraatsstudenten) psychologisch en organisatorisch met raad en daad bijstonden. Geert, Bruce, Sandra, Philippe, Ariane, Sylvia en

Virginie: het was telkens een verademing om op een open en constructieve manier met jullie te kunnen samenwerken; iets waarvan ik hoop dat het ook in de toekomst verder zal mogelijk zijn. Ook de mensen van het secretariaat, rotsen in de branding in dit chaotische wereldje, kan ik niet anders dan mijn eeuwige dank verschuldigd te zijn. Hoe zou het geweest zijn als jullie deskundigheid mij niet tussen de Scylla en Charybdis van dreigende vergeten afspraken en deadlines zou hebben geleid? Katia en Mieke, Isabelle, Leen, Nathalie en Liesbeth, dank voor de administratieve ondersteuning, dossierwerk, het telefonisch afschepen van (de natuurlijk totaal imaginaire) vervelende patiënten maar vooral ook de ondersteuning en vriendschap.

Zijn voor de inhoud van dit werk de patiënten van een onschatbare waarde geweest, het welslagen van dit doctoraat zou nooit gelukt zijn zonder de onvoorwaardelijke steun en ondersteuning van het thuisfront. Paps en mams, zonder jullie zou er van geneeskunde nooit sprake geweest zijn. En zonder de regelmatige “en hoe zit het nu nog met het doctoraat?” vraag zou deze thesis nu misschien nog niet voor jullie liggen. Dank voor alle kansen die jullie mij hebben gegeven, het vertrouwen, de liefde en ondersteuning.

Iets meer dan een jaar geleden hield ik een wezentje in mijn armen. Klein en onschuldig, nog niet goed gewassen en dus wat pluizig, met een grote open blik naar de wereld. Lieve Simon, kleine kabouter, jij hebt me de voorbije maanden op je eentje geleerd waar niemand tot dan ooit in was geslaagd: relativering. Je pretoogjes en guitige lach doen me glimlachen, je kan me de stress van het alledaagse doen vergeten en doen opgaan in die kleurrijke, sprookjesachtige wereld die jij thuis noemt. Ik hoop dat ik een stukje van jouw aanstekelijke spontaneïteit – zoals hameren op het toetsenbord terwijl papa’s grote boekdocument nog openstaat – kan bewaren, koesteren en uitstralen.

De dagen tijdens en na een doctoraat zijn lang, de avonden – en soms ook de nachten – kort. De stress die gepaard gaat met voordrachten, deadlines, bepaalde niet-ideale werkomstandigheden, durven zich dan weleens meester maken van mijn anders zo vrolijke zonnetje-in-huis karakter. Liesbeth, mijn liefste, ik besef maar al te goed – zeker als ik zit te wachten tot je thuis komt van de volleyball ;-) – hoe vaak ik je heb laten wachten of alleen gelaten heb de voorbij jaren door overwerk, thuiswerk of ander gezwerk. Je onvoorwaardelijke steun, belangstelling en oppeppers wanneer nodig hebben de voorbije jaren - en in het bijzonder de laatste maanden - gezorgd voor telkens dat ene lichtpuntje dat nodig was om er weer even tegenaan te kunnen. Hoewel ik je niet kan beloven dat alles vanaf nu rustiger wordt – je kent me, ook al kan een pak stress nu achterwege worden gelaten – neem ik me voor om ook in de toekomst maximaal te blijven genieten van ons gezinnetje...

Zo, beste lezer, bent u aan het einde van “het grote boek” gekomen, een boek waarvan ik hoop dat ik er in de toekomst nog vele bladzijden mag aan toevoegen. Ik dank u voor uw aandacht, interesse (al dan niet geveinsd ;-) en bijbestellingen van bijkomende exemplaren. Until we meet again...

Gent, 26 december 2008